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MEMBRANE FORMATION BY PHOSPHOLIPIDS IN WATER–TERTIARY BUTANOL SYSTEMS

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MEMBRANE FORMATION BY PHOSPHOLIPIDS IN WATER–TERTIARY BUTANOL SYSTEMS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. W. DEN BOER, HOOGLERAAR IN DE FACULTEIT DER LETTEREN, TEN OVERSTAAN VAN EEN COMMISSIE UIT DE SENAAT TE VERDEDIGEN OP DINSDAG 7 JULI 1964 DES NAMIDDAGS TE 2 UUR

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Promotor: Prof. Dr. H. L. BOOIJ

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Aan Ännchen

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CHAPTER I.

INTRODUCTION.

1. THE PROTOPLASM MEMBRANE.

The composition of the cytoplasm of living cells is entirely different from that of the surrounding medium (see e.g. Gamble, 1950). The predominant cation in the intracellular fluid, for instance, is potassium, in the extracellular fluid sodium. The principal extracellular anions are chloride and bicarbonate, the intracellular anions phosphate and proteins. It is known that constancy of the chemical composition and of organization of the living cell is essential to its normal physiological behaviour. To preserve this constancy independent of fluctuations in the composition of the outer medium, there must be a barrier between the cytoplasm and the medium, a surface layer which decreases or eliminates the free diffusion of ions. On the other hand, the living cell is in a metabolic steady state. The metabolic exchanges with the environment with maintenance of the chemical composition of the cell have led to a number of permeability theories (Hokin and Hokin in Annual Review of Biochemistry Vol. 32, 1963).

One of the basic problems of permeability is the not yet elucidated architecture of the protoplasmic membrane.

One of the first theories put forward concerning the chemical nature of the protoplasmic surface layer was the lipoid theory of Overton (1895), whose observations on the permeability of plant cells led him to state that the rate of penetration of a substance into the cell could be related to its lipoid-solubility: e.g. ethers, ketones, and aldehydes penetrate very rapidly, while the more water-soluble ethylene glycol, glycerol, and erythritol penetrate more slowly.

This lipoid theory still plays an important part in the more modern conceptions of the structure of the protoplasmic membrane (Booij and Bungenberg de Jong, 1956; Stein and Danielli, 1956; Finean, 1956).

The classic contribution to the problem of the chemical composition and structure of the protoplasmic membrane is that of Gorter and Grendel (1925, 1926). Employing the measuring technique developed by Langmuir (1917), their experiments led to a conception of a structural lipoid layer. These authors extracted the lipids from erythrocytes and spread them as a monolayer on the surface of a Langmuir trough. Relating the area of this monolayer to the surface area of the erythrocytes from which the lipids were extracted, they concluded that the cell membrane probably consisted of a double layer of lipid molecules. Analysis of the composition of the extracted lipids showed the presence of cephalin, lecithin, sphingomyelin, and cholesterol. In terms of this finding, Gorter and Grendel visualized the structure of the cell membrane of an erythrocyte as a double layer of oriented phospholipid molecules as illustrated in Fig. 1.

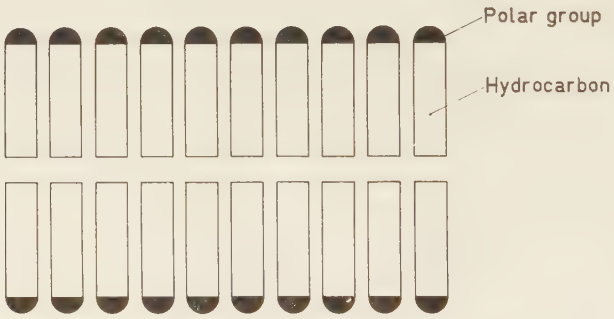


Fig. 1. Gorter & Grendel's (1926) model of the possible structure of the protoplasmic membrane of erythrocytes consisting of a double layer of phospholipid molecules.

More recently, an extensive study of the composition of the lipids of erythrocytes from various animals has been given by De Gier (1960).Depending on their origin, the erythrocytes showed the lipids lecithin, cephalin, sphingomyelin, and cholesterol as main components in different percentages.

Over the years, various assumptions have been made about the morphological and chemical composition of the protoplasmic membrane, based on various biological experiments (Booij and Bungen-

berg de Jong, 1956, p. 131). However, until the introduction of the electron microscope, only indirect data concerning the existence of a protoplasmic membrane structure were available. Electron microscopical studies have revealed not only the existence of a membrane structure at the boundary of the cell but also the existence of various other membrane structures, either in the cytoplasm (the endoplasmic reticulum, Palade and Porter (1954), Palade (1955), or in cytoplasmic organelles (mitochondria, Palade (1953)); chloroplasts, Hodge (1959). Sjöstrand (1959) referred to this feature as follows:

"When dealing with the cytoplasm (in electron microscopical studies), one gets the impression that the construction of a surface membrane with certain properties represents one of the first steps in the creation of life, and that this principle has been applied in a rather monotonous way by nature". The structural similarity of all these membranes has led to the unit membrane concept (Robertson, 1959, 1961, 1964).

From electron-microscopical investigations on the formation of myelin in chick embryonic nerves, Geren (1954) concluded that the myelin sheath in adult nerves originated from the Schwann's satellite cells. The multi-layered sheath represents the elongated and spirally-wound cell membrane of the Schwann cells around the axon. This discovery is of great importance, since it correlated the presence of large amounts of lipids (sphingomyelin, cerebrosides, lecithin, cephalin, and cholesterol) in the myelin sheath with the occurrence of these lipids in cell membranes.

Without giving additional evidence, we may assume that the general occurrence of lipids in living cells, particularly phospholipids and cholesterol, is related to the protoplasm membrane structure. With Booij (1962) we may now pose the following, most pertinent, questions about the architecture of the living cell's membrane: "How is it possible that a lipid structure survives at the interface of two phases (cytoplasm and medium), both containing water in great amounts? Is it necessary to assume a protecting network (e.g. consisting of proteins) built in or attached to the lipid layer?"

This question forms the central theme of this thesis. Some thirty years ago, Bungenberg de Jong and Westerkamp (1931, 1932)

observed peculiar phenomena in coacervate drops of a phospholipid system which are not found in the simple coacervation of detergents with salts. These peculiar phenomena have been related to an arrangement of the phospholipid molecules at the surface of the drops, forming an interface between two aqueous media; the inner medium of the drop and the outer medium.

In Chapter II (Fig. 7) we will mention phenomena shown by a commercial lecithin preparation in an aqueous - tertiary butanolic medium. These phenomena could be measured conductometrically and suggested the presence of structural units in the solution. Studying these phenomena with an improved technique and with phospholipids freshly prepared from biological material, we can investigate whether phospholipids can arrange themselves at a water-water interface without the aid of a covering by other substances.

This does not exclude the presence of other macromolecular substances such as proteins, polyphosphates, carbohydrates, etc. (Booij 1962). These covering substances may play a functional role superimposed on the structure formed by the phospholipids.

2. THE CLASSIFICATION OF THE LIPIDS.

The chemical classification of the lipids differs from that of proteins and carbohydrates in that the latter can be considered as polymers of more or less similar structural units, whereas the lipids have no characteristic element or chemical groups. This is explained historically, since the lipids were defined only by their insolubility in water and their solubility in the so-called "fat-solvents" such as diethyl ether, chloroform, and benzene, rather than by their chemical composition and reactivity. Thus Deuel (1951), following the original classification of Bloor (1925), includes in this class such derivatives as carotenoids and vitamins D, E, and K. Among the waxes are classified the esters of cholesterol and of vitamins A and D.

In our classification we refer only to those compounds which can be considered as derivatives of fatty acids, the waxes being excluded. These derivatives can be divided into two main groups: the simple lipids (neutral fats) and the complex lipids (in which other components are substituted). For a general review of the subject, the

reader is referred to papers by Wittcoff (1951), Hilditch (1956), Lovern (1957), Hanahan (1960), and Bloch (1960).

In Fig. 2 we have tried to show schematically the composition of the main constituents found in this group of the lipids. In the centre is placed the group of the fatty acids which corresponds with the general formula R-COOH. These fatty acids are combined with the radially-placed constituents to build up a certain subclass of lipids, indicated by the name placed at the outer arc of a segment.

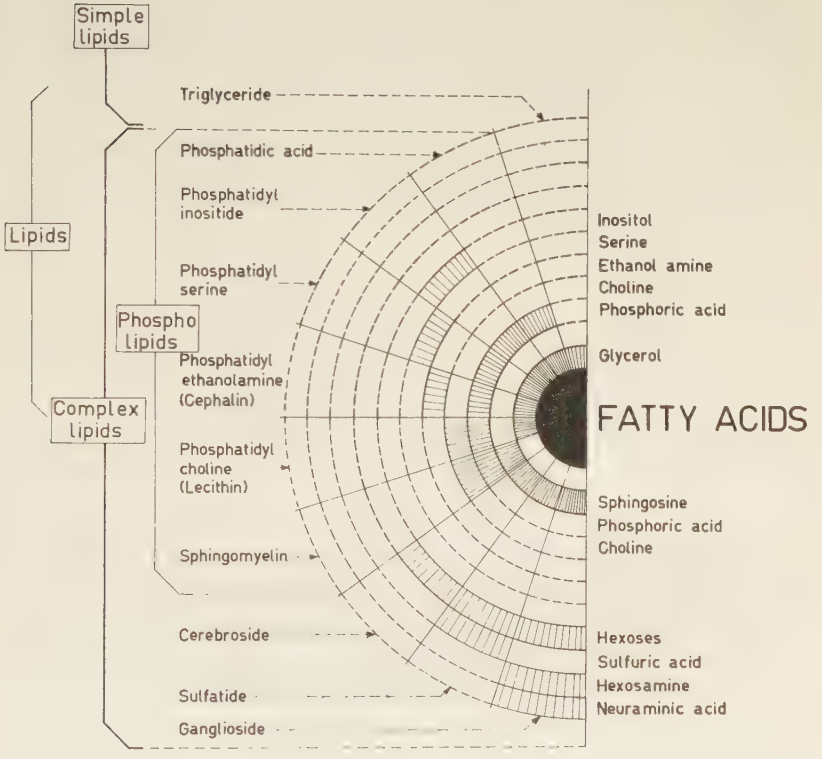


Fig. 2. Classification of lipids as derivatives of fatty acids.

In the simple lipids, three fatty acids are esterified with glycerol, while in the case of the complex lipids, two or only one fatty acid is esterified. Considering the great variety of fatty acids that occur

naturally (Boldingh, 1963) in combination with different alcohols which in their turn can be replaced by different groups, the complexity in chemical structure whithin this class is understandable.

3. THE PHOSPHOLIPIDS.

Among the lipids, the phospholipids are considered to be the most important in membrane structures. The common factor in this group is the presence of phosphorus in the otherwise widely differing composition of the molecules. Considering the alcoholic moiety of the molecule, a subdivision can be made into phospho-glycerides and phospho-sphingosides. From these basic molecules, various molecules are derived by substitution. These derivatives occur in widely differing quantities in biological material. Generally (depending on the source), phosphatidyl-choline, phosphatidyl-ethanolamine, and sphingomyelin are the most abundant, the others (see Table I) forming a minor part.

Table I.

Subdivision of phospholipids.

Phosphatidyl-choline (lecithin)
Phosphatidyl-ethanolamine (cephalin)
Phosphatidyl-serine
Plasmalogens, differing from the preceding lipids by containing a vinyl-etherical linked aldehyde instead of a fatty acyl group
Phosphoinositides, mono-, di-, and tri-phosphoinositides
Phosphatidic acid
Cardiolipin
Lyso-compounds, differing from the above by the absence of one fatty acid in their molecule
Sphingomyelin.

Sphingomyelin also belongs to a subclass with great variety, namely the sphingo-lipids. However, the physico-chemical properties of sphingomyelin are more comparable to those of lecithin, owing to their similar hydrophilic (choline) part in the molecule.

In this thesis the investigations are restricted to the main, naturally-occurring phospholipids, lecithin, sphingomyelin, and cephalin, the structural formulas of which are given in Fig. 3.

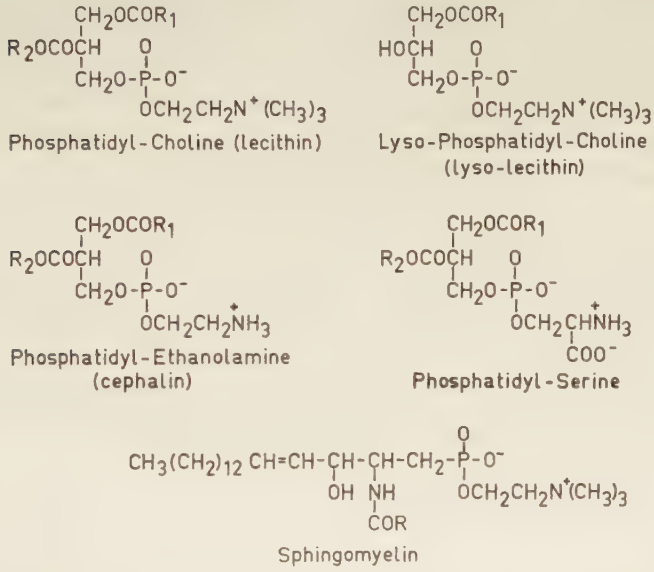


Fig. 3. Formulas of the phospholipids mentioned in this investigation.

4. CONSIDERATION ON THE PROPERTIES OF PHOSPHOLIPIDS.

In spite of the diversity of their chemical composition, there is a great similarity in molecular configuration of the phospholipids. All components consist of a hydrophilic charged group and one or two hydrophobic long carbon chains. For sphingomyelin one of these chains is not an introduced fatty acid but originates from sphingosine. All these molecules have an amphipatic character (a hydrophilic plus a hydrophobic part) which is also found in simple de-

tergents (e.g. sodium dodecyl sulfate, sodium oleate, etc.). This amphipathy is responsible for many analogous properties shown by these different molecules:

1. They can be spread as a monolayer on a water surface.
2. Under suitable circumstances they show the formation of myelinic figures in contact with water.
3. Their aqueous solutions can under suitable circumstances show a high viscosity, elasticity, and coacervation.

According to Staudinger (1929), the amphipatic substances can be classified as "association colloids" in which the colloid particles make their appearance by a reversible association of single, much smaller molecules. The association colloids have been intensively studied by Booij (1949) and Booij and Bungenberg de Jong (1956), who emphasized their importance as biocolloids.

a. **Monomolecular film formation.**

In the first section of this chapter, the spreading of monolayers was mentioned in connection with the investigations of Gorter and Grendel (1926). Leathes (1923, 1925), in studying the formation of monolayers by lecithin, found the area of each lecithin molecule in cross-section to be 56.6 Å^2. Since the area occupied by a fatty acid was estimated at about 21 Å^2, he concluded that the hydrophilic part of the lecithin molecule occupied a larger area than the sum of the two fatty acids. Turner and Watson (1930) spread sphingomyelin and estimated the cross-sectional area to be 42 Å^2. From this it must be concluded that the carbon chains are packed closely together. The sphingomyelin film thus exists in a more condensed state than the lecithin film.

In view of the presence of cholesterol in biological material containing lecithin, Leathes studied the behaviour of mixtures of these substances. Addition of cholesterol decreases the area initially occupied by lecithin. The action of cholesterol was given some quantitative basis by the work of De Bernard (1957), who studied mixed monolayers of lecithin and cholesterol under a surface pressure of 5 dynes/cm. Under these circumstances he estimated two optimal mixing ratios: A, 3 mol. lec. : 1 mol. chol. and B, 1 mol. lec. : 3 mol. chol., both ratios showing a relatively condensed area.

The influence of pH alterations and the presence of salts in the aqueous phase can be generally understood in terms of the Coulomb forces between the hydrophilic groups. Suppressed dissociation or salt formation led to more condensed films in the case of monolayers of fatty acids (Adam and Miller, 1933; Matalon and Schulman, 1947; Harkins, 1952; Havinga, 1952).

Recently, Van Deenen and co-workers (1962) published an interesting investigation on mono-molecular layers of synthetic phospholipids. They concluded from their experiments that variations in the fatty acid composition, particularly unsaturation but also decrease of the chain length (e.g. from C_{18} to C_{15}), led to a less condensed film, thus to a greater cross-sectional area per molecule.

b. **Formation of myelinic figures.**

One of the most remarkable and interesting microscopical observations is the behaviour of a smear of phospholipids on a slide in contact with water. The generation in the aqueous phase of little sprouts which grow out to elongeted, flexible threads, and their continuous movements, rounding off, confluence, and new formation is an ever-fascinating spectacle.

The name "myeline" was given to these figures by Virchow (1854), because of their resemblance to the myelin sheath of nerve fibres. Leathes (1925) studied the formation of these figures with a lecithin preparation, and found that their formation was inhibited by calcium ions but that this effect could be nullified by additions of cholesterol.

The property of forming myelinic figures is, as mentioned above, not restricted to the phospholipids; other substances or mixtures of substances may behave in the same way. This was shown for lysolecithin and cholesterol, sodium cetylsulfate and cholesterol, cetylalcohol and digitonine by Dervichian (1946). Ekwall and co-workers (1956) investigated the myelinic growth of mixtures containing sodium laurate and decanol. The higher fatty acids can be forced to produce myelinic formations by addition of sufficient amounts of potassium chloride.

c. Aqueous systems of association colloids.

Increasing the amount of a detergent (e.g. potassium oleate or sodium dodecyl sulfate) in an aqueous solution, we observe at a given concentration striking alterations in various physical properties (e.g. surface tension, osmotic pressure). These alterations, clearly shown graphically by Preston (1948), are due to the formation of aggregates – called micelles – in the solution. The concentration at which they appear is called the critical micelle concentration or CMC. At this concentration the upper limit of disturbance in the internal solvent structure is reached and the hydrocarbon chains are expelled (Oosterbeek, 1962). This causes an arrangement in which the hydrocarbon chains adhere to each other while the hydrophilic parts of the molecules are turned to the water. The existence of such associations above the CMC was recognized by Mc Bain (1913). Once united in aggregates of molecules, the molecular interactions will come into being, first between the hydrophobic parts of the molecules (London van der Waals forces) and second between the hydrophilic parts of the molecules (Coulomb-forces). This is illustrated in Fig. 4.

Fig. 4

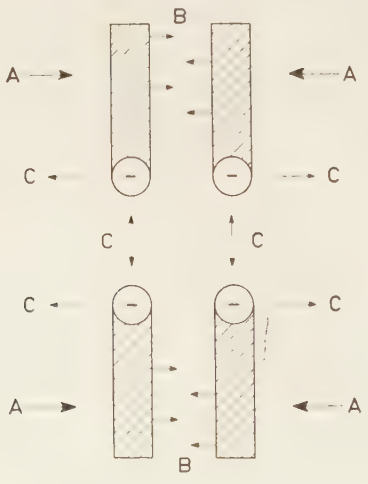


Fig. 4. The factors determining the shape of the detergent micelle. A — the expelling factor of the medium, B — the London van der Waals forces between the carbon chains, and C — the Coulomb forces between the hydrophilic groups.

The morphology of the micelle has been a matter of discussion for many years. Hartley (1936) proposed the occurrence of a spherical type of micelle, whereas McBain (1944) accepted a lamellar type as well. Harkins (1952) considered the existence of a cylindrical type of micelle. Gradual addition of an appropriate salt (e.g. KCl) to a soap solution (e.g. 1% K-oleate) gives rise to systems with different properties: viscous systems, elastic-viscous systems, coacervated systems, and a final system showing a small smectic (liquid crytalline) upper layer and a large aqueous lower layer. According to Booij's theory (1949), these subsequent altered properties are due to subsequent changes in the shape of the micelle: spherical, cylindrical, plate-like, and sandwich forms. These various types of micelles are evoked by the subsequent neutralization of the repulsive Coulomb forces by the added salt.

In attempting to place the phospholipids in this sequence we must be aware that the charged group of these molecules is composed of a negative and a positive part (Fig. 3). To arrange these kinds of molecules in a plate micelle, no repulsive forces need be eliminated since the positive part of one molecule will act with the negative part of the adjacent molecule. Thus, instead of a repulsive action the charged hydrophilic part of the molecule helps to form a stable layer. Here, the neutralizing counter-ions are already fixed in the molecules themselves. From this it will be clear that the phospholipids do not readily form coacervates, elastic-viscous systems, or isotropic solutions; they are, as it were, predestined for the smectic state. To realize other systems the mutual ionic attraction must be weakened. This can be done, as shown by Bungenberg de Jong, De Bakker and Andriesse (1955a, b) by screening one of the charges by a counter-ion showing a very high affinity for this group. These authors studied the influence of increasing amounts of sodium salicylate, which shows a high affinity for the quaternary ammonium group, on a suspension of smectic lecithin drops. They were able to realize coacervation, elastic-viscous systems, and non-elastic systems. With this investigation the close relation between the colloid chemistry of detergents (cationic or anionic association colloids) and of amphoionic association colloids was clearly demonstrated.

d. **Influence of alcohols on coacervated systems.**

The action of substances on different systems of association colloids (elastic-viscous systems or coacervated systems) becomes generally understandable if this action is interpreted as a strengthening or weakening of the intramolecular forces in the plate micelle and/or an influence between the micelles. Studying the action of added substances on coacervated systems, this means that a strengthening of the intramolecular forces will lead to a decrease of the coacervate layer (condensing action) and the weakening of these forces will be reflected in an increase of the coacervate layer (opening action).

From the work of Rosenthal (1939) we reproduce two graphs indicating the action of the lower alcohols, including tertiary butanol, on oleate coacervates.

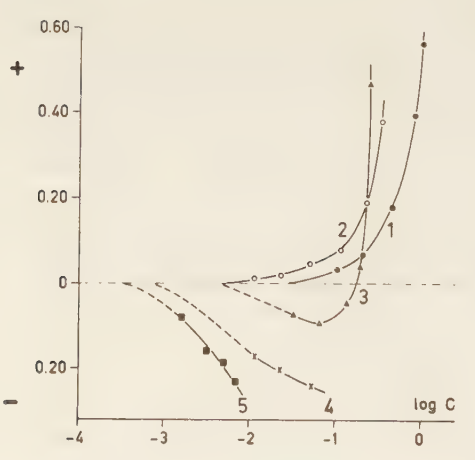


Fig. 5.

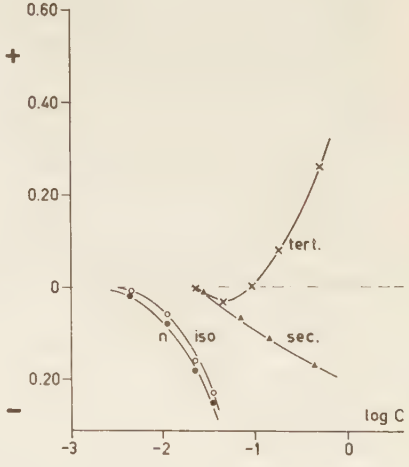


Fig. 6.

Influence of alcohols on the volume of an oleate coacervate.
Abscissae: logarithm of the alcohol concentration in mol/L (log C).
Ordinates: logarithm of the relative change in coacervate volume (log V/V_0).

Fig. 5. Influence of methanol (1), ethanol (2), propanol (3), butanol (4), and amylalcohol (5).

Fig. 6. Influence of isomeric butanols, normal butanol (n), isobutanol (iso), secondary butanol (sec), and tertiary butanol (tert), according to Rosenthal.

Fig. 5 shows clearly the inversion of the action in this homologous series with an increase in the number of carbon atoms, ethanol having an opening action and butanol acting as a condensing agent. The action of propanol is an example of inverse action corresponding to increasing concentration of the added substance. Fig. 6 shows the influence of branching of the carbon chain and displacement of the hydroxyl group at an equal number of carbon atoms. The action of tertiary butanol then resembles the action of propanol. Study of the action of tertiary butanol on potassium laurate and on potassium ricinoleate revealed only a strong condensing action, indicating that the action of this alcohol is inversed by shortening and increased hydrophility of the carbon chain of the "substrate".

e. P-coacervation.

The condensing action of amyl alcohol can be used to produce coacervation in a system containing insufficient amounts of salt to cause coacervation. When amylalcohol is added in sufficient amounts in the presence of not too low a salt concentration, a peculiar coacervation may be produced. Observation of this coacervation under the microscope shows drops with peculiar surface phenomena, absent in the normal coacervation. This peculiar coacervation is called P-coacervation (P-originally standing for phosphatide), the more common coacervation being called O-coacervation (O = ordinary). This phenomenon, which was observed some thirty years ago by Bungenberg de Jong and Westerkamp (1931, 1932) while studying phospholipid systems, is not restricted to phospholipid systems but is a morphological phenomenon also shown by other association colloids under suitable conditions. In Fig. 7 we have reproduced a picture of the peculiar drops as revealed by microscopical observation, according to Bungenberg de Jong and co-workers.

Bungenberg de Jong, De Bakker and Andriesse (1955a, b) studied the action of lower alcohols on egg-phospholipid suspensions in more detail. This suspension contains drops of phospholipids consisting of coiled myelinic figures. The study of this suspension provides information about the action of different substances on the smectic state of phospholipids. The action of methanol, ethanol and

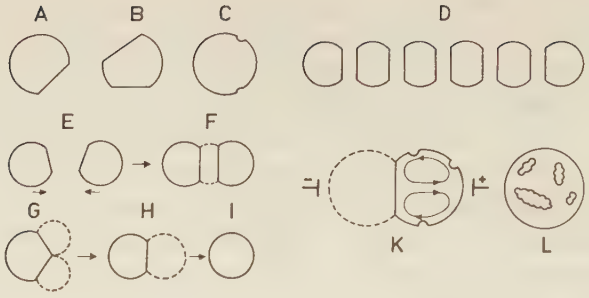


Fig. 7. Morphological peculiarities of P-coacervates (after Bungenbery de Jong and co-workers).

A, B, C drops with sideways-attached vesicles, the protruding vesicle not being visible.

B - I A mechanical disturbance may rupture the vesicle wall, after which the coacervate drop immediately rounds off.

E - F Formation of a mechanical unit, consisting of two coacervate drops and a common vesicle. D a larger similar unit.

L Chain of vacuoles separated by invisible septa.

G - H Sideways fusion of two vesicles.

K Behaviour of a negatively-charged coacervate drop in a direct-current field. The moving boundary transports small sideways attached vesicles to the cathode side of the drop where they fuse to one vesicle which gradually increases in size. With positive coacervate drops the movement of the boundary is just the other way around and the large vesicle develops at the anode side.

propanol converts the suspension into a clear solution; the higher homologues altered the suspension into a coacervated system which on microscopical observation shows the peculiarities of P-coacervation. To cause P-coacervation with n-octanol, n-nonanol, or n-decanol, the temperature must be made unsuitably high. It was found that this elevation in temperature could be replaced by the addition of lower alcohols. We have already seen that these lower alcohols act as opening substances on ordinary oleate coacervates. At high temperatures P-coacervation was also found to result from tertiary butanol additions to the phospholipid suspension.

CHAPTER II.

PRELIMINARY INVESTIGATIONS AND FORMULATION OF THE PROBLEMS.

In 1957, Bungenberg de Jong and Davids described an optical phenomenon observed on dilution of a clear solution of purified commercial "lecithin" in tertiary butanol with distilled water. At a given alcohol concentration (about 25%) the clear solution became blue-opalescent. It appeared that this phenomenon depended not only upon the concentration of tertiary butanol but also on the temperature: at higher temperatures, the transition occurred at lower alcohol concentration. Another phenomenon was also observed at higher temperatures: the transition "point" expanded to a transition "region". The borderline between the clear solution and the opalescent system splits into two diverging lines enclosing a transitional region with a glassy turbidity. This relationship between the concentration of tertiary butanol and temperature and the appearance of a transitional region is shown graphically in Fig. 8.

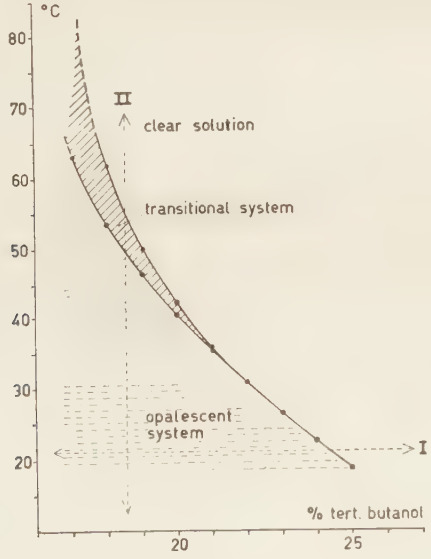


Fig. 8. Influence of the tertiary butanol concentration and of the temperature on the appearance of a lecithin solution (Merck's preparation).

To obtain more information about this phenomenon, Bungenberg de Jong and Lexmond (1957a, b, c) approached it by way of conductometric titration. They placed a dipping electrode in the clear alcoholic lecithin solution and recorded the change in resistance on dilution of the solution with distilled water at 22° C. In doing this the system passes along Arrow I in Fig. 8 from the clear solution into the opalescent system. At a given dilution, a sudden rise in resistance was found, and this rise appeared to coincide with the transition point, as illustrated in Fig. 9 by A.

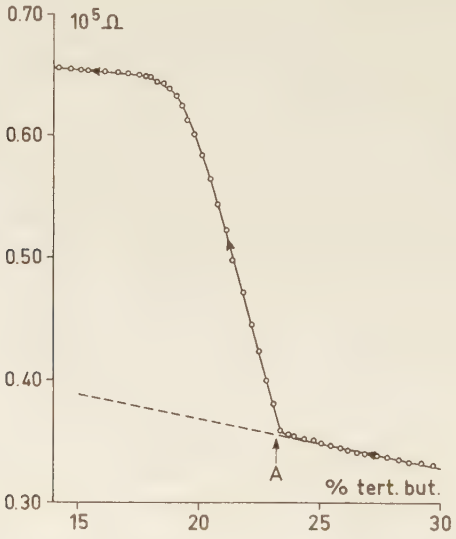


Fig. 9. Conductometric titration curve of a lecithin solution (Merck's preparation) in tertiary butanol.

The experiment shown graphically in Fig. 9 was performed without addition of electrolytes. Considering the different (inversed) downard slope of both the titration curve of the medium and the clear branch of the curve (right to arrow A in Fig. 9) and their position at different resistance values (e.g. at 25% tertiary butanol: medium $2.34 \times 10^5\Omega$, lecithin solution $0.34 \times 10^5\Omega$), Bungenberg de Jong and Lexmond concluded that the phospholipid contributes to the conductivity of the solution. On raising the conductivity by

addition of 4 meq NaCl/L it appeared that the slope of the right branch of the graph coincides fairly well with the corresponding part of the blank curve. These measurements also revealed increase of the resistance at conductometrical titration of an initially clear phospholipid solution in tertiary butanol.

Bungenberg de Jong and his collaborators concluded from both their optical and their conductometric titration investigations that the increase in resistance of the commercial lecithin system at gradual lowering of the tertiary butanol concentration must be caused by structural units of lecithin.

The results obtained by conductometric titration measurements of the commercial lecithin preparation can be summarized as follows:

1. The onset of the increase of the resistance appeared to be practically independent of: the lecithin concentration; the addition of cetyl alcohol and cholesterol.
2. The increase of the resistance appeared to be independent of: the NaCl concentration, the $CaCl_2$ concentration (above 1 mmol/L) and the pH (up to pH 8.2).
3. The increase of the resistance appeared to be dependent on: the pH (above pH 8.2), the addition of Na-laurylsulfate, cetene, oleylalcohol, n-decanol, cetylalcohol, and cholesterol.

1. DIFFICULTIES PRESENTED BY THE CONDUCTOMETRIC TITRATION OF COMMERCIAL LECITHIN SOLUTIONS.

The conductometric titration method provided several important data, as listed above. However, the method shows limitations which are inherent in the titration technique:

1. The reversibility of the phenomenon cannot be studied. (The increase in resistance follows dilution with distilled water; a technique with subsequent extraction of the added water is impossible).
2. The concentration of lecithin decreases when water is added.
3. The value of the resistance depends strongly on mechanical agitation; stirring of the solution results in a relatively lower resistance. Since the titration method requires stirring after each

addition of water to obtain a homogeneous solution, the resulting resistance values are unreliable.

4. At a given high dilution with water, a sudden sharp drop in resistance is observed, i.e. the resulting system becomes unstable.

2. THE THERMO-CONDUCTOMETRIC METHOD.

In view of these limitations of the titration method, another way of measuring the phenomena occurring in the lecithin solution was considered. Fig. 8 shows the relationship between the temperature and the tertiary butanol concentration for a solution of a commercial lecithin preparation. The appearance of this solution (clear, turbid, or opalescent) is determined by these two variables. With the conductometric titration method, we follow arrow I (Fig. 8); the temperature is fixed and the concentration of tertiary butanol is gradually lowered. However, it must also be possible to lower the temperature at a fixed concentration of tertiary butanol. Starting with a clear solution at a higher temperature the system would then pass along arrow II in Fig. 8. The advantages of such an experimental approach are obvious:

1. The experiment is performed in a system with constant concentrations of all constituents.
2. Stirring can be avoided.
3. The experiment can also be performed in the opposite direction; a cooling period can be followed by a heating period.

This method, which we will call the "thermo-conductometric method", should make it possible to study the phenomena with greater precision and accuracy and to attack the problems left unsolved by the conductometric titration method.

3. FORMULATION OF THE PROBLEMS.

a. Problems which can be solved by an improved technique.

1. Is it possible to realize a complete reversibility of the phenomenon?
2. What is the influence of stirring the solution?
3. What is the influence of the phospholipid concentration of the solution?

b. **Problems which are inherent in the measured substance.**

1. What is the composition of commercial lecithin?
2. Which component of this preparation is responsible for the increase in resistance?
3. Does a pure lecithin preparation behave in the same manner? If not, are the impurities a *conditio sine qua non*?
4. If pure lecithin causes an increase in resistance, what factors influence this increase, e.g.: the action of lecithinase A, pH variations of the medium, salt additions, additions of cholesterol, etc.?
5. Do fresh lecithin preparations originating from biological material show the same phenomena?
6. If this phenomenon is not restricted to commercial lecithin, what behaviour is found in other membrane constituents such as sphingomyelin or cephalin?

In view of these questions, it is clear that the first problem encountered after the construction of appropriate measurement equipment is the preparation of phospholipids from biological material in order to estimate whether the phenomena have a more general significance.

The investigation of the preparation of natural phospholipids resulted in a rapid method delivering greater quantities in a fairly pure state. This part of our study has been reported in an Appendix in order to maintain a logical arrangement of the other investigations.

CHAPTER III.

MICROSCOPICAL INVESTIGATIONS.

1. OBSERVATIONS IN A LECITHIN SYSTEM DURING GRADUAL ALTERATION OF THE TEMPERATURE.

Bungenberg de Jong and Davids (1957a) have made a microscopical study of the behaviour of a purified commercial lecithin preparation in tertiary butanol which also contained cholesterol. At very slow heating of an initial optical clear opalescent lecithin-cholesterol system (O, in Fig. 10) they observed the appearance of peculiar coacervate drops (1, in Fig. 10) which subsequently changed at further gradual heating, as shown in Fig. 10, states 1 to 6. When the temperature was lowered by spontaneous cooling, changes were observed as shown in Fig. 10, states 6, 7, 8, 9, and 11.

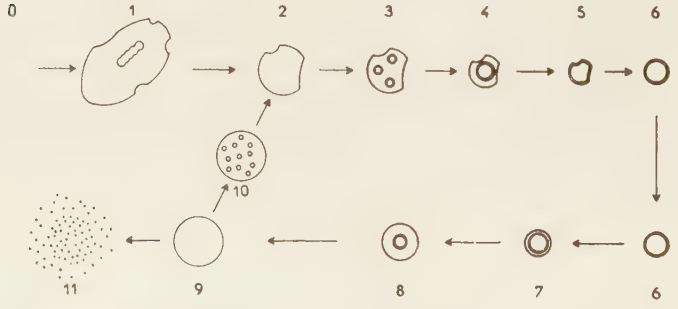


Fig. 10. Morphological observations in a cholesterol – containing phospholipid system in 22.8% tertiary butanol during slow heating (upper row) and subsequent spontaneous cooling (lower row), according to Bungenberg de Jong and Davids.

The observations made during slow heating and subsequent cooling can be summarized as follows (the numbering corresponds to Fig. 10):

1. Patches of a coacervate appear, containing articulate vacuoles. The typical shape of the vacuoles indicates that this coacervate belongs to a peculiar type.

2. Gradually the coacervate shrinks.

3. On further shrinking, another type of inclusions appears, containing higher concentrations of phospholipids.

4. The inclusions coalesce into one large drop.

5-6. The surface of the included drop coalesces with the outer surface.

6, 7, 8, 9, and 11.

These figures represent the phenomena observed on gradual cooling of the system. When cooling is stopped at state 9 by switching on the heat supply, state 2 is realized via state 10.

Restricted by the capacity of the heating table they employed, Bungenberg de Jong and Davids did not achieve clear solutions at higher temperatures in the sequence of their observations. Their observations are pertinent with regard to the transitional region. However, the initial appearance of the peculiar coacervate drops may point to the peculiar morphological state of the phospholipid in the opalescent region.

2. OBSERVATIONS IN A LECITHIN SYSTEM AT RELATIVELY CONSTANT TEMPERATURE.

In this section we will describe the changes that take place in a lecithin solution in tertiary butanol on gradual dilution with water. These changes were examined in a hanging drop containing a fresh lecithin preparation, extracted from hen's egg-yolk and purified by column-chromatography as described in the Appendix.

Equipment.

For this hanging drop technique we used the microslide equipment as shown in Fig. 11, consisting of a nylon plate (P) 80 × 30 × 5 mm containing a well. The well is 20 mm in diameter and 3 mm deep, with straight sides and a flat glass bottom (B), and can be closed with a cover glass (C). Two small holes (H) lead from the edge of the slide into the well, and in one of them a syringe needle

(N) is fixed through which solutions can be introduced into the closed well. The lecithin solution is applied as a large drop (D) to the cover glass, which is then inverted and placed on the nylon plate as shown in Fig. 11.

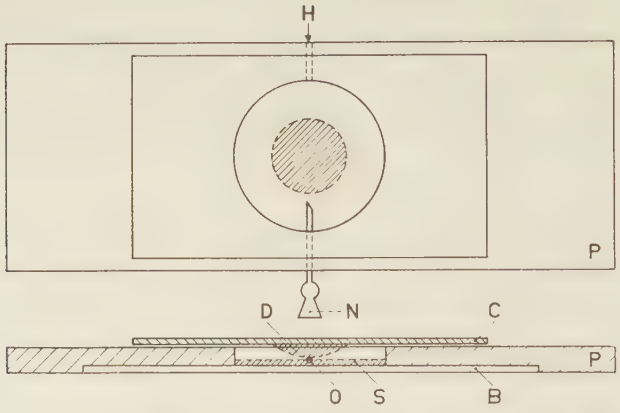


Fig. 11. Micro-slide equipment used for the observation of changes taking place in a lecithin system (D) in 45.5% tertiary butanol on gradual dilution with water.

Prior to use, a thin layer of silicone grease is smeared on the slide around the well in order to seal the space between cover glass and slide. After placing the slide under the microscope, the margin of the drop is brought into focus; the microscope light is then switched off to prevent heating of the bottom of the well. Through the syringe needle, distilled water is brought into the well until a thin layer (S) covers the bottom; the microscope light may then be switched on again.

Observations.

After a few minutes a very fine granulation appears at the margin of the drop. This process of granulation proceeds gradually until it reaches the centre of the drop; in the meantime the marginal granules become coarser and change, via small dense droplets into droplets with double-refractive surface layer. From these droplets small, thin myelinic sprouts grow out very rapidly. The compactness of these sprouts is rapidly lost and vesiculation occurs on their outer

surface, producing a membrane structure. These observations reveal the following sequence from the centre of the drop to its margin:

1. Clear centre.
2. Very fine granulation.
3. Coarse granules.
4. Dense droplets.
5. Droplets with double-refracting surface.
6. Myelinic sprouts.
7. Swollen myelinic figures.
8. Vesiculation of the myelinic figures.
9. Membrane structures.

Fig. 12 shows a photograph of states 3 tot 9.

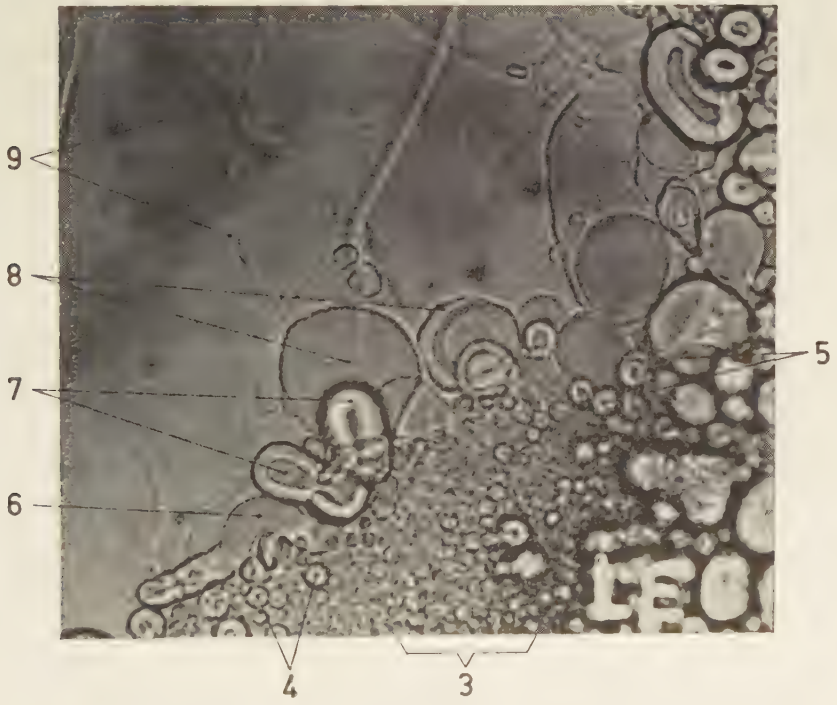


Fig. 12. Photograph of phenomena (states 3 to 9, see text) occurring in a hanging drop containing 1.5% lecithin in tertiary butanol (45.5%) on gradual dilution with water. The centre of the drop lies in the direction of the lower right-hand corner.

Discussion.

The causative factor involved in these phenomena must be the gradual decrease in the tertiary butanol concentration. The bottom of the small chamber is heated slightly by the light of the microscope while the cover glass maintains a lower temperature. This difference in temperature causes a gradual transport of water from the bottom of the well to the cover glass. When water condenses into the hanging drop, the decrease in the tertiary butanol concentration will be more rapid along the margin of the drop than in its thicker centre. In this way a dilution gradient is realized from the margin to the centre of the hanging drop.

Since we started with a clear lecithin solution, the initial fine granulation must be the microscopical representation of entering the macroscopically-observed transitional region. The observed sequence of phenomena thus shows microscopically the conversion of a clear lecithin solution into an opalescent system on gradual dilution with water. However, we have of course no information about the variation of the tertiary butanol concentration from the centre to the margin of the hanging drop. Since the phenomena occur in rather rapid succession, we get at best an approximate idea of what goes on. Nevertheless, we may conclude that the opalescent region represents a very peculiar state, resembling a foam. The surface membranes of the bubbles appear in the two-dimensional microscopic picture as a thread-like structure consisting of lecithin molecules, while the inner and outer spaces of these bubbles are filled with aqueous tertiary butanol, corresponding to the air in a foam.

In Chapter VI, we will examine these structural questions in more detail by submitting phospholipid - tertiary butanol systems to thermo-conductometric measurements.

CHAPTER IV.

THE EQUIPMENT FOR THERMO-CONDUCTOMETRIC MEASUREMENTS AND ITS EMPLOYMENT.

In thermo-conductometric measurements the following procedure is employed. A turbid suspension of phospholipid in a mixture of tertiary butanol and water, to which a salt has been added, is heated until a clear solution is obtained. The temperature of this clear solution is gradually decreased to 20° C at a slow, constant rate; the resistance of the solution is measured at regular temperature intervals. The variation in resistance can be plotted as a function of the temperature. The solvent of the system must be protected against evaporation to keep the experimental conditions constant. Subsequently, a curve for the resistance is obtained for the process in the opposite direction with rising temperatures.

From this description we derive the following requirements:

a. The phospholipid system must be in a closed vessel provided with a thermometer and electrodes connected to an apparatus for the measurement of the resistance.
b. This vessel must be placed in a bath the temperature of which can be regulated continuously.

1. MEASUREMENT VESSELS.

The measurement vessels used in this investigation are shown in Fig. 13. Their volumes are 25 ml (vessel I) and 5 ml (vessel II). Both vessels are provided with an extension tube E in which the system can expand when the temperature of the full, closed vessel is raised.

During cooling and heating there is a temperature gradient between the centre of the system and the wall of the vessel. Since the resistance is a function of the temperature, this radial temperature gradient will be reflected in a radial resistance gradient. To minimize

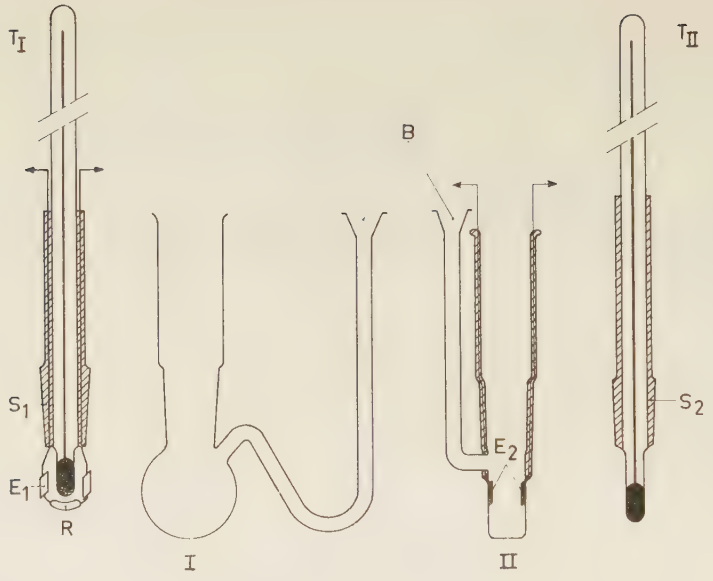


Fig. 13. Vessels for the measurement of the resistance of phospholipid solutions. Stopper S_I belonging to vessel I (volume 25 ml) carries a thermometer T_I and the electrodes E_I; stopper S_{II} belonging to vessel II (volume 5 ml) carries only a thermometer T_{II}, the electrodes E_{II} being sealed in the wall of the vessel.

the chances of hysteresis in the temperature-resistance reading during cooling and heating, it is essential that the thermometer be centred between the two electrodes. To achieve this, the ground-glass stoppers S_I and S_{II} were perforated and the thermometers fixed in the holes with Araldit.*)

The electrodes are 0.6 cm squares of platinized platinum. In the case of the spherical vessel I, the electrodes are attached to the stopper by means of short fused lengths of platinum wire and held in position, to prevent accidental disturbance, by a glass rod R sealed between them. To restrict the resistance measurements to the central part of the vessel, the outer sides of the electrodes are insulated with a sealed glass layer. The electrodes of Vessel II are

*) Araldit was kindly put at our disposal by the office of CIBA Limited in The Netherlands, Arnhem.

sealed in the wall of the vessel, the connecting platinum wires being led through the wall. Resistance is measured with a Philips conductance meter ("Philiscop II", G.M. 4144/01.).

2. EQUIPMENT FOR LINEAR COOLING AND HEATING.

The measurement vessel is placed in the middle of a water bath which can be heated and cooled linearly at a velocity of 0.2° C/min by varying the heat-supply in combination with constant cooling with running tap-water of constant temperature. Figure 14 gives a diagram of the equipment.

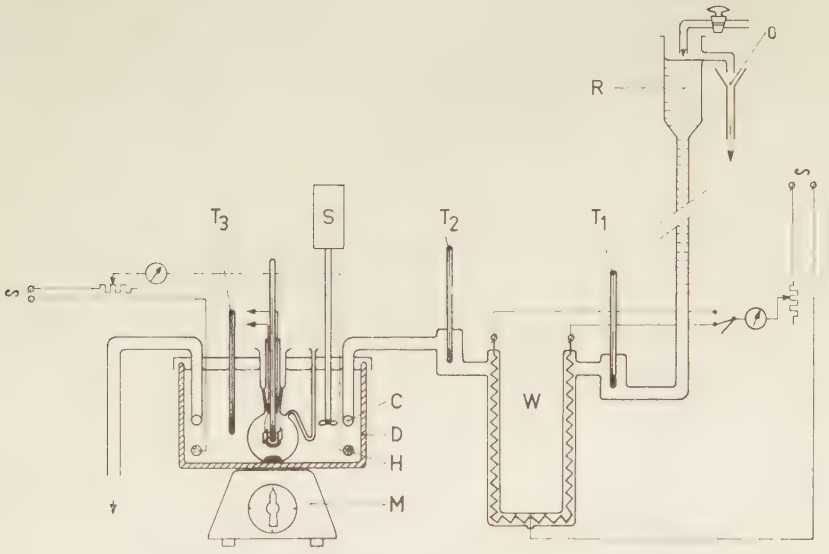


Fig. 14. Equipment for linear cooling and heating of the solution in the measurement vessel placed in the middle of water bath D.

The bath consists of a Pyrex vessel (D) filled with 1800 ml of distilled water. This vessel is provided with: a heating loop (H); a cooling loop (C); and a stirrer (S). To limit temperature exchange with the surroundings, the sides and bottom of the bath are insulated with foam plastic and the bath covered with a plexiglass plate. Stirring in the measurement vessel is made possible by mounting the bath on a magnetic stirring apparatus (M). The heating loop (H)

consists of a coil of 250 Watt heating wire in a perforated glass stocking. The heating capacity of this wire is regulated with a transformer. Cooling loop (C) is made of copper and connected with reservoir (R) mounted 2 meters above the water bath. The reservoir is filled to a constant level with running tap-water, the level being maintained by means of an overflow (O). This constant difference in height insures a constant flow of cooling water. In the connecting glass tube, a heating wire (W) is mounted in order to maintain the cooling-water at a constant temperature (16° C) independent of the initial temperature of the tap-water.

To obtain a cooling system with a velocity of 0.2° C/min we determined the relation between the bath temperature and the voltage supplied to the heater by a Variac transformer; constant cooling with water at 16° C was applied. These values are listed in Table II.

Table II.

Voltage of the heater.		Temperature of the water bath in ° C.
150	—	59.0
140	—	54.0
125	—	47.7
100	—	37.7
90	—	34.0
75	—	30.1
50	—	24.3
20	—	20.6

From these values the graph in Fig. 15 was derived by plotting the square of the voltage against the obtained temperature of the water bath. Since the heat developed is proportional to the square of the voltage, we find in the Figure a linear relationship between the corresponding temperature. The resulting graph, however, represents a relation independent of the time necessary to reach a constant temperature at a given voltage of the heating loop. To use

this graph for a linear cooling and heating scheme, we had to find a way of changing the heat-supply step-wise, or in other words, a time scheme for adjustment of the voltages of the heating wire to obtain a cooling or heating rate of 0.2° C/min.

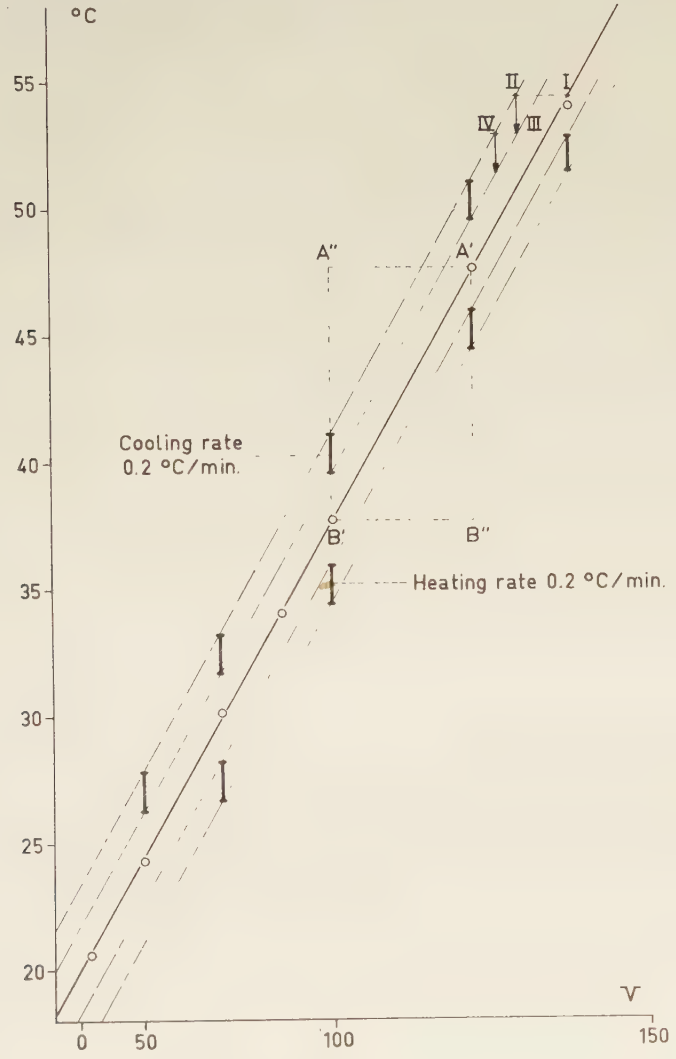


Fig. 15. Relation between the square of the voltage of the heating loop (250 Watt) in the water bath and the resulting constant temperature of the bath (drawn line). For the explanation of the parallel lines see text.

Starting from situation A′ (see Fig. 15), voltage 125, temperature 47.7° C, we adjusted the voltage at 100 (situation A″). Cooling from A″ (47.7° C) to B′ (37.7° C) is initially very rapid, but this rate decreases when lower temperatures are reached. During this process, time and temperature were registered; it was found that from 41.1° C to 39.6° C, cooling at a rate of 0.2° C/min was achieved. The experiment was performed in reverse for the heating process. Starting from situation B′ (37.7° C at 100 V), the voltage was adjusted at 125 V (B″) and the temperature course from B″ to A′ was registered. It appeared that from 44.4° C to 46.0° C a heating of 0.2° C/min was achieved. Similar experiments were done at other voltages and other temperatures; the regular periods found are indicated in Figure 15 by heavy black vertical lines. These periods lie between parallel lines defining regions of constant cooling and constant heating rate at the corresponding relation ° C/Voltage. To realize a continuous cooling or heating rate of 0.2° C/min over a certain temperature range (e.g. between 60° C and 20° C) the voltage of the heating wire must be readjusted step-wise during this course in accordance with the appropriate zone in Fig. 15.
For example:

The temperature of the water bath is raised by adjusting the voltage of the heating wire to 150 V, and shunting the cooling loop. At 60° C the cooling water (16° C) is led through the water bath. After a constant situation is reached (I in Fig. 15) the heating wire is set at a lower voltage corresponding to the point at which the horizontal line drawn from point I to the left intersects the outer line of the cooling zone (point II). At this voltage we permit the bath to cool (at a rate of 0.2° C/min) till a temperature corresponding to the intersection of the vertical line at the adjusted voltage and the inner line of the cooling zone (point III). At this temperature the voltage is readjusted to a value given by the intersection of the horizontal and the outer line of the cooling zone (point IV), and so on. Following this procedure the cooling zone is traversed in descending steps. The same procedure must be followed for the regulated heating course according to the relations represented by the heating zone in Fig. 15. By adjusting the voltage of the heating wire step-wise at higher voltages, the heating zone is traversed upwards, also at a constant rate of 0.2° C/min.

3. MEASUREMENTS ON A BLANK SYSTEM.

Regulating the heat supply according the scheme developed in the preceding section, we recorded the resistances of a blank system (without phospholipid) during regulated cooling and heating.

Composition of the blank system:

10.0 ml tertiary butanol 91%,

25.0 ml sodium chloride 20 meq/L,

15.0 ml double distilled water.

The measurement vessel (I or II) is provided with a small, iron-core stirring rod, filled with the system up to the joint, and then closed with its special ground-glass stopper. The surplus of solution above the joint is removed with filter paper. The solution in the vertical part of the extension tube E is adjusted to a constant low level by removing the surplus with a long hollow needle. The vessel is then placed in the water bath and the electrodes connected to the Philoscop to record resistance. After closing the bath with the plexiglass coverplate, the stirrer S is mounted and started. Next, the temperature is raised quickly, to 55° C for example. (During this rapid rise, the cooling is cut off by a shunt.) At this high temperature, the regulated slow decrease in temperature (0.2° C/min) is started and continued until 20° C is reached. Then the reverse course is followed by increasing the heat supply. The resistance readings, taken at whole degrees, are listed in Table III. The measurement of the blank was repeated three times to check reproducibility; the differences between corresponding readings were not greater than 1%. From Table III we derived Fig. 16 by plotting the resistance readings against the corresponding temperature.

With lowering of the temperature, the resistance increases because the velocity of the conducting ions decreases. At the same time, the viscosity of the solution increases. These factors determine the upward movement of the resistance curve at low temperatures. The cooling and heating curve lie close together in Fig. 16, indicating that this equipment provides very good reversibility.

Table III.

Resistance values at various temperatures during regulated cooling and heating.

Regulated cooling		Regulated heating	
temperature °C	resistance Ω	temperature °C	resistance Ω
51.0	363	20.0	783
50.0	369	21.0	757
49.0	378	22.0	735
48.0	385	23.0	713
47.0	394	24.0	691
46.0	401	25.0	670
45.0	410	26.0	654
44.0	419	27.0	635
43.0	429	28.0	618
42.0	437	29.0	599
41.0	446	30.0	583
40.0	456	31.0	568
39.0	468	32.0	552
38.0	480	33.0	540
37.0	491	34.0	524
36.0	505	35.0	512
35.0	515	36.0	500
34.0	528	37.0	489
33.0	541	38.0	480
32.0	556	39.0	464
31.0	572	40.0	456
30.0	587	41.0	446
29.0	604	42.0	435
28.0	621	43.0	426
27.0	639	44.0	416
26.0	660	45.0	409
25.0	680	46.0	401
24.0	700	47.0	391
23.0	720	48.0	383
22.0	740	49.0	376
21.0	765	50.0	368
20.0	785		

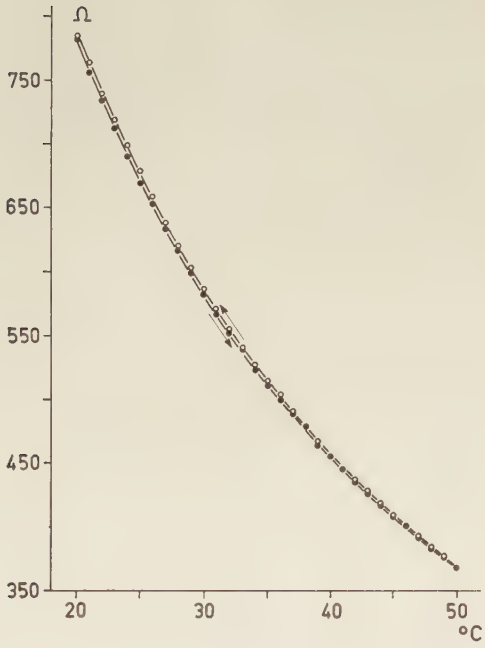


Fig. 16. Correlation between resistance and temperature in a blank system containing 18.2% tertiary butanol and 10 meq NaCl/L, under regulated cooling and heating.

CHAPTER V.

THERMO-CONDUCTOMETRIC INVESTIGATIONS.

This chapter reports the investigation of the physico-chemical properties of phospholipids in aqueous solutions containing tertiary butanol. The greater part of the investigation concerned lecithin prepared from hen's eggs, although the properties of other phospholipids (egg cephalin and beef spinal-cord lecithin, sphingomyelin, and cephalin) were also studied. In the first section of this chapter we include, for the sake of comparison, a conductometric titration in addition to the thermo-conductometric measurement.

A. MEASUREMENTS ON A COMMERCIAL "LECITHIN" PREPARATION.

The lecithin solution was obtained from a Merck preparation ("lecithin ex ovo puriss.") which we purified of neutral fats, fatty acids, and other substances according the technique described in the Appendix. After measurement of the dry weight, a 3% stock solution was made in 91% tertiary butanol. This stock solution was used to prepare the sample solutions, both containing 10 meq NaCl/L.

Methods.

a. The conductometric titration.

A 100 ml vessel holding 10 ml of the "lecithin" stock solution and 20 ml of a solution containing 15 meq NaCl/L is placed in a water bath (temp. 25° C) mounted on a magnetic stirring device and provided with a glass stirring rod with an iron core. In a perforated rubber cover-plate are mounted a thermometer and a dipping electrode connected to the Philoscop GM 4144 (Philips) conductometer. From a burette, a solution of 10 meq NaCl/L is added step-wise, following a rigid time scheme for addition, stirring, and measure-

ment. After addition of about 10 ml, the solution in the vessel becomes opalescent and when stirred shows whirling streaks. The additions and measurements are continued until 30 ml has been added from the burette; the tertiary butanol concentration is then reduced from 30.33% to 15.17% and the phospholipid concentration from 1% to 0.5%. To exclude the effects of variations due to equipment, technique, or the solutions employed, the measured resistances are divided by the resistances found by corresponding measurement of a blank system (without "lecithin").

b. Thermo-conductometric measurement.

These measurements were performed on the same lecithin stock solution according the technique described in Chapter IV for the measurement of the blank system. The solution to be measured is prepared by pipetting into a 100 ml Erlenmeyer flask:

10.4 ml stock solution,

25.0 ml NaCl 20 meq/L,

15.0 ml double-distilled water.

Part of this opalescent phospholipid system is transfered to vessel I (Fig. 13). The rest of the solution is poured into a small flask, provided with a ground-glass stopper, which is also placed in the water bath. This second vessel enables us to check the composition of the solution during the course of the experiment by paper chromatography.

The temperature of the water bath is increased until the phospholipid system becomes a clear solution (52° C), after which temperature and resistance are recorded during a regulated cooling and heating course.

Results.

For the evaluation of the results of the measurements, the resistances recorded at whole degrees are divided by the corresponding values obtained from the blank systems. The quotients Ω/Ω_0 (resistance factors) can then be plotted against the corresponding temperatures. The resulting graphs are shown in Figs. 17 and 18.

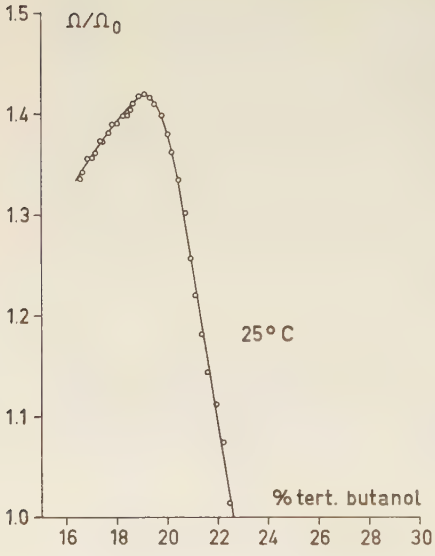


Fig. 17.

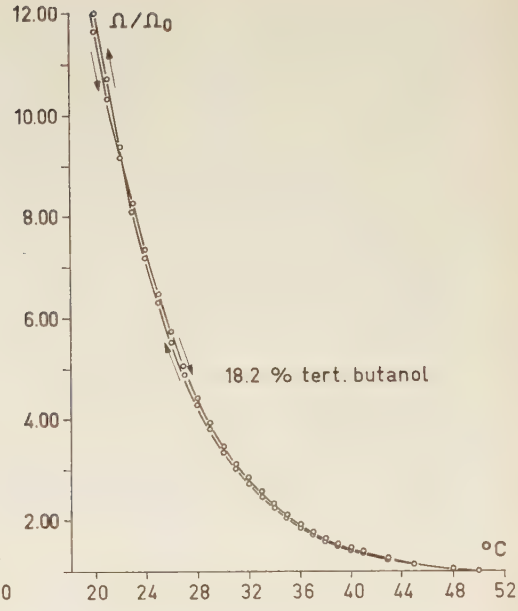


Fig. 18.

Measurements of purified lecithin ex ovo puriss. (Merck).

Fig. 17. Resistance factor curve obtained with the conductometric titration method.

Fig. 18. Resistance factor curve obtained with the thermo-conductometric method.

In examining Figs. 17 and 18 the following points come to the fore:

1. The resistance factors of the thermo-conductometric method are much higher than those obtained with the conductometric method.
2. Fig. 17 shows a maximum, whereas in Fig. 18 the curves follow a smooth and regular course in both directions.
3. The reversibility of the phenomenon is practically complete with the thermo-conductometric method, the ascending and descending curves lying close together.

Discussion.

The thermo-conductometric method is superior to the conductometric titration method. The response of the resistance factor to

physico-chemical alterations of the phospholipid system is much greater with the thermo-conductometric technique, and is also completely reversible.

Since these results were obtained with a purified commercial lecithin preparation, the question arises: which component of this preparation is responsible for the rise of the resistance? To answer this question, we analysed the solution by paper chromatography according the technique described in the Appendix. To detect small concentrations of the components, a series of solutions of increasing concentration was applied on the paper. Fig. 19 shows the resulting chromatogram after staining with the tricomplex staining bath (see Appendix).

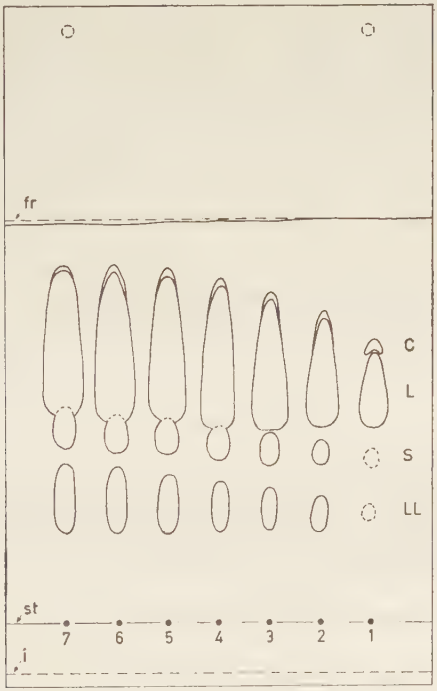


Fig. 19. Chromatogram of the purified commercial lecithin preparation measured by the thermo-conductometric method. The figures under the starting points (on line st) indicate how many times the solution was spotted on the paper. i-immersion line of the solvent; fr-front line of the solvent; C-cephalin; L-lecithin; S-sphingomyelin; LL-lysolecithin. Solvent: diisobutylketon – acetic acid – water, 50 : 25 : 5, by volume.

From Fig. 19 it is evident that lecithin is the major component of this preparation although cephalin (phosphatidyl-ethanolamine), sphingomyelin, and lysolecithin (lyso-phosphatidyl-choline) are present and may contribute to the phenomena. Another question remains: do the peculiar properties of this preparation originate from chemical alteration of the substances (e.g. of the fatty moieties) occurring during its commercial manufacture. Such alteration is suggested by the strong yellow-brown colouration and by the presence of fatty acids in the commercial product.

B. MEASUREMENTS ON FRESH PREPARATIONS.

The physio-chemical study of natural complex lipids has been hindered by the great difficulties encountered in the isolation and purification of the individual components in sufficient amounts. Taking biological material as our source, we worked out a method yielding phospholipids in amounts adequate for our experimental purposes. In view of the lability of some phospholipids, which causes an increase of the negative surplus charge (Bungenberg de Jong and Hoogeveen, 1961c), the method had to be rapid and constituents with anti-oxydant action (Vonk, 1961; Pitt and Morton, 1962) had to accompany the phospholipids as long as possible. We developed an extraction method for fresh material; without prior fractionation, the total extract was separated into its main constituents by column-chromatography.

The development of such a method was essential to the subsequent investigations on the structural properties of the phospholipids. The resulting technique is applicable to all kinds of tissue including whole blood. The development of the extraction, separation, and purification methods is described in the Appendix to this thesis.

I. EXPERIMENTS WITH HEN'S EGG-LECITHIN.

1. The temperature-tertiary butanol diagram.

The physico-chemical properties of freshly-prepared, purified lecithin might be quite different from those shown by the commercial

preparation. Since it is known from preliminary experiments that the occurrence of an opalescent system is correlated with an increase of the resistance factor, we first investigated the optical behaviour of the fresh egg-lecithin.

Equipment and method.

A 500 ml beaker filled with distilled water is provided with an iron-core stirring rod and placed on a magnetic stirring device. A heating loop of spiral resistance wire mounted in a perforated glass stocking is introduced into the water. The temperature of the water is gradually raised; cooling occurs automatically when the current is switched off. A 10 ml test tube provided with a small glass iron-core stirring rod and a thermometer fitted into the ground-glass stopper of the tube is mounted in the bath. Lecithin solutions of various tertiary butanol concentrations are introduced into the test tube. The optical changes in the solution in response to raising and lowering its temperature are recorded for the various temperatures of the solution.

Solutions.

The composition of the solutions is shown in Table IV.

Table IV.

	ml	ml	ml	ml	ml	ml	ml
6.4% lecithin in 91% tert. but.	0.54	0.54	0.54	0.54	0.54	0.54	0.54
Tert. butanol 91%	0.40	0.45	0.50	0.55	0.60	0.70	0.80
NaCl 20 meq/L	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Aqua bi-dest.	1.60	1.55	1.50	1.45	1.40	1.30	1.20
% tert. butanol	16.38	17.29	18.20	19.11	20.02	21.84	23.66

Anticipating the results, we prepared these solutions according the requirements of thermo-conductometric measurements:

a. The conductivity of the solutions is raised by adding 2.5 ml NaCl 20 meq/L.

b. The calculated volume of the lecithin dry weight (0.04 ml) is added to the 0.50 ml of the stock solution. This is done to obtain tertiary butanol concentrations corresponding to those of the blank solutions (without lecithin).

The lecithin was a 6.4% stock solution in 91% tertiary butanol prepared from column fractions of a fresh egg-yolk extract according the technique described in the Appendix. To relate the results of this and further investigations to the phospholipid composition of the stock solution, we checked it by paper chromatography (described in the Appendix). For this purpose, the 6.4% stock solution was diluted ten times with tertiary butanol 91%, the final concentration corresponding to that of the solutions mentioned in Table IV. This solution was spotted on $SiCl_4$-impregnated paper (see Appendix) in a series of increasing concentrations by spotting 1, 2, 3, 4, 5, and 6

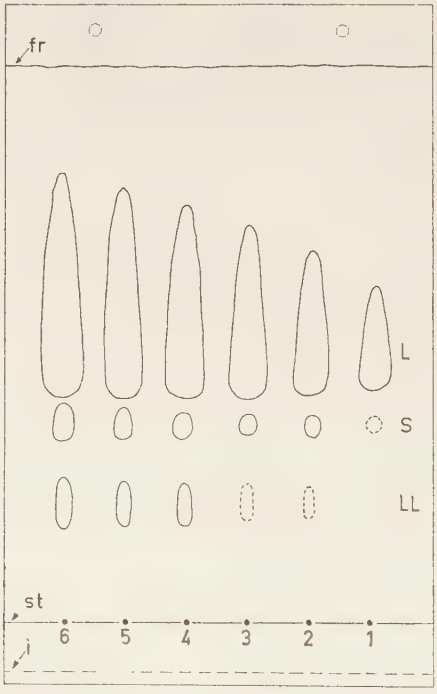


Fig. 20. Chromatogram of a ten-times diluted 6.4% lecithin stock solution in 91% tert. butanol. The figures under the starting points (on line st) indicate how many times the solution was spotted on the paper. i-immersion line; fr-front line; L-lecithin; S-sphingomyelin; LL-lysolecithin.

times. The higher concentrations enabled us to detect smaller concentrations of impurities. After staining according to the tricomplex staining procedure (see Appendix), we obtained the chromatogram shown in Fig. 20.

Although this fresh lecithin preparation is free of the cephalin fraction, it still contains very small amounts of sphingomyelin and lysolecithin.

Results.

Heating and cooling of the solutions in Table IV showed that all opalescent solutions could be converted into clear solutions by raising the temperature. After calculation of the mean values of the temperatures at which the optical alterations occurred, these values were plotted against the percentage of tertiary butanol of the given solution.

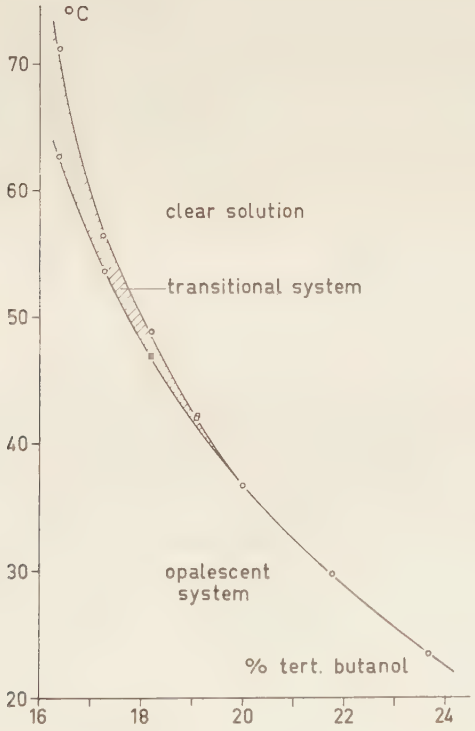


Fig. 21. Temperature – tertiary butanol diagram of solutions containing 0.64% of hen's-egg lecithin.

Fig. 21 shows the resulting graph. From 16.38 to 23.66% tertiary butanol we observe a descending curve as a border line between the clear lecithin solution and the opalescent system. From 20.02% to lower concentrations the curve splits off into two diverging lines with a transitional region between them.

Discussion.

The optical phenomena of this purified fresh lecithin preparation are analogous to the phenomena observed in the commercial lecithin preparation shown in Fig. 8, derived from the work of Bungenberg de Jong and Davids (1957).

We conclude that this behaviour is not a reflection of chemical alterations produced during the manufacture of commercial lecithin.

2. The resistance factor curve of hen's-egg lecithin.

The analogous optical phenomena found in the fresh hen's-egg lecithin and the commercial "lecithin" led to the further investigation of the fresh preparation by means of the thermo-conductometric method.

Equipment and method.

See Chapter IV.

Solutions.

On the basis of the optical temperature-tertiary butanol diagram we selected a tertiary butanol concentration of 18.2% for these experiments. The following solution was prepared:

5.4 ml 6.4% lecithin stock solution in 91% tertiary butanol,

5.0 ml 91% tertiary butanol,

25.0 ml NaCl 20 meq/L,

15.0 ml Aqua bi-dest.

The blank system consisted of a corresponding solution without lecithin.

Results.

From the registered resistance values for both the lecithin system and the blank the resistance factors were calculated. These resistance factor – temperature relations are plotted in Fig. 22.

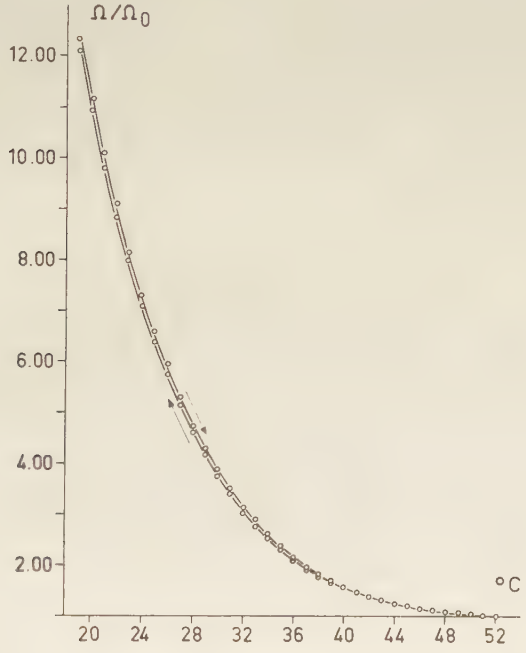


Fig. 22. Resistance factor curves of purified hen's-egg lecithin showing reversibility of the phenomenon.

The resistance factor curves lay close together, indicating that the phnomenon is practically thermo-reversible. The high resistance factors of this fresh preparation at 20° C show great resemblance to those obtained with the commercial preparation. We must thus conclude that the optical and the resistance alterations are indentical for both preparations.

3. Mechanical alterations of the resistance factor curve.

A preliminary investigation (Bungenberg de Jong and Hoogeveen 1958b) into the influence of mechanical disturbance on the resistance factor of a commercial lecithin preparation has shown that even slight stirring of the solution is rapidly reflected in a strong decrease of the resistance factor. Since we found that besides lecithin this preparation also contained cephalin, sphingomyelin, lysolecithin, and in addition strongly-coloured substances it was necessary to determine whether these contaminations acted as labilizing factors in the rapid reaction to slight mechanical agitation. This could be partially checked in the fresh lecithin preparation because cephalin and coloured substances were absent.

Method.

To study the stability we started with a high-resistance system obtained by regulated cooling of a lecithin system identical to that used in the preceding experiment. After cooling to 20° C the small stirring rod in the measurement vessel was switched on and the effect on the resistance was recorded for 20 minutes. Stirring was then stopped and regulated heating applied. A second experiment concerning constant stirring during regulated heating and cooling was also done.

Results.

After calculating the resistance factors they were plotted in Fig. 23 as a function of the temperature. The curves of the first experiment are labelled I and of the second, II.

Curve I shows that the ultimate resistance factor (11.16) of the system decreases very rapidly on stirring (about 50% in 2 minutes) followed by a more gradual decrease until a constant resistance is reached ($\Omega/\Omega_0 = 3.71$). During the subsequent heating in the absence of stirring there is no return to the original higher resistance factors. The resistance factor curve of the experiment with constant stirring (II) lies much lower, but over the whole range shows complete reversibility.

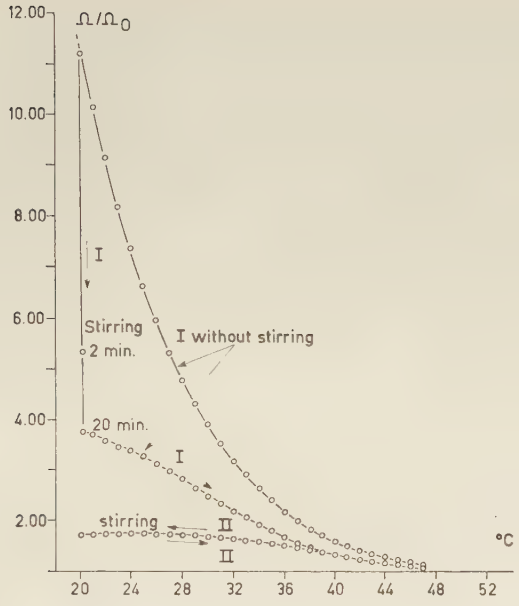


Fig. 23. Influence of stirring on the resistance factor curves of a hen's-egg lecithin system. Curves labelled I: intermediate stirring, Curves labelled II: constant stirring during regulated cooling and heating.

Discussion.

The resistance factors of hen's-egg lecithin are found to be very rapidly reduced by mechanical agitation. The effect of stirring on a previously-formed high-resistance system is less than the effect of stirring during the formation of higher resistance factors. This indicates that the primary structures which cause the initial increase of the resistance factor are readily disrupted by stirring. The remaining resistance factor of the stirred high-resistance system must be due to fragments of the original structures.

The results of these experiments correspond with those of experiments previously done with the commercial lecithin preparation. We may therefore conclude that the other constituents of the commercial lecithin preparation either have no influence on the properties of the lecithin or that their effects cancel each other.

4. Influence of snake venom on a lecithin system with a high resistance factor.

The hemolytic action of snake venom is well known. This phenomenon is explained by the action of the lecithinase A content of this venom on the lecithin in the cell membranes of the erythrocytes. The lecithin is converted into lysolecithin and free fatty acids. This conversion disrupts the membrane structure, so that hemoglobin and other cell constituents leak out into the surrounding medium. The question was whether injection of snake venom lecithinase would affect the membranous structures present in a solution of lecithin in a water – tertiary butanol system. We used a 0.1% solution of snake venom (Crotalus adamanteus) in a phosphate buffer (pH 7.4).

Experimental procedure.

After regulated cooling of a lecithin system identical to that used in the preceding experiments ($\Omega/\Omega_0 = 11.47$), a very thin ployethylene tube was inserted into the bulb-shaped measurement vessel (II) via the extension tube. This thin tube was connected to a syringe and 1 ml of the lecithin system was carefully removed. The tube was then closed, the syringe detached, and the sample of the lecithin solution brought into in a test tube. This solution was later chromatographed. We then brought into the syringe successively, 1 ml snake venom sol. and 2 ml of the lecithin solution from the measurement vessel. This mixture was injected slowly into the measuring vessel while avoiding agitation. After 40 hours the stirrer was switched on for 2 minutes, followed by regulated heating without stirring.

At 55° C stirring was repeated for 5 minutes and the system left to stand for 50 hours. After this period (totalling 90 hours), regulated cooling and heating was performed; the composition of the system was then checked by paper chromatography. For graphical evaluation of the results of this experiment, we performed an analogous experiment without lecithin in the same way.

Observations.

After injection of the snake venom the high resistance of the original system remained unaltered. Twenty-four hours later the re-

sistance was still the same but a clear region was observed in the lower part of the opalescent solution below the electrodes. After 40 hours the system on the bottom of the vessel was quite clear but this area did not reach the electrodes, which may explain why the resistance of the system was still practically the same. At this time we decided to stir, since it appeared that the diffusion of the snake venom was too slow. The resistance was recorded after 2 minutes of stirring and was found to have dropped to a very low value. Regulated heating was then performed, and it was found that the conversion temperature originally situated at 49.8° C had shifted to the much lower temperature of 33.6° C.

Results.

Thermo-conductometric measurements.

In Fig. 24 we have plotted the whole procedure in terms of the temperature/resistance factor relation.

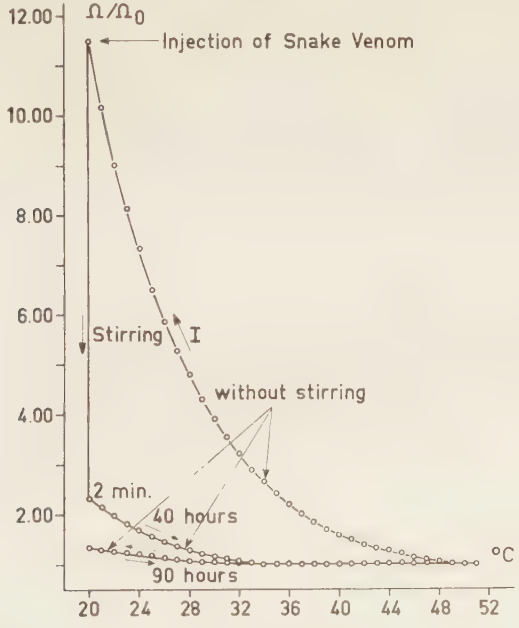


Fig. 24. Influence of the action of injected snake venom on a high-resistance system of hen's-egg lecithin.

Regulated cooling led to a system with a high resistance factor (11.47), and since the injection of the snake venom did not effect the resistance, this factor remained constant during the next 40 hours. Stirring after that time reduced the resistance factor to about 20% of its original value. The curve of the heating course represents very low values of the resistance factor, and these values are much lower after 90 hours.

Chromatograms.

Before injecting the snake venom into the lecithin system, 1 ml of the system was removed for a paper chromatogram, made according

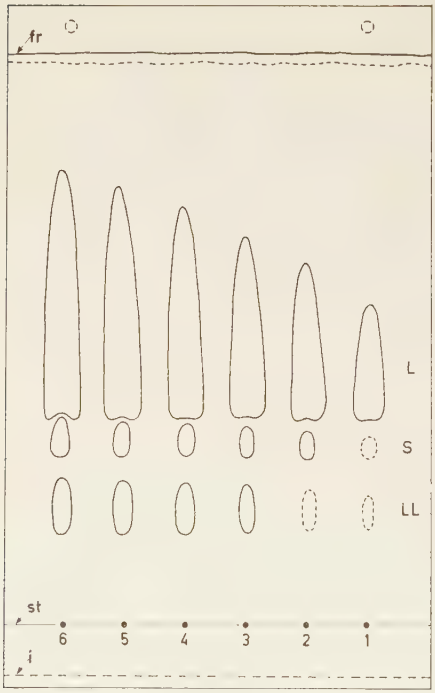


Fig. 25.

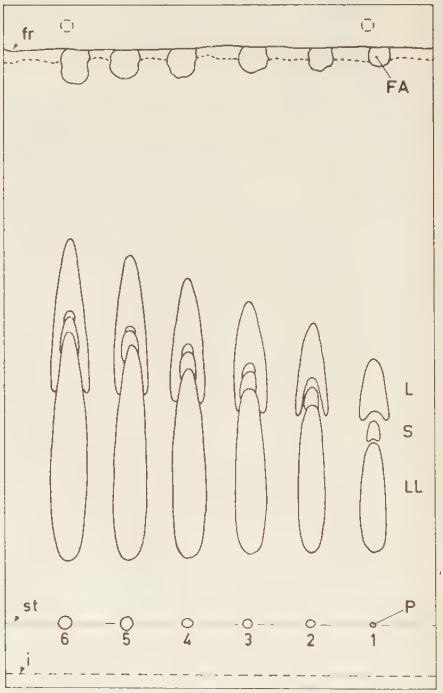


Fig. 26.

Chromatograms showing the influence of snake venom on a lecithin system. Fig. 25 converted in 90 hours to a system chromatographed in Fig. 26. The figures below the starting points indicate how many times the solution was applied. fr.-front line; st-starting line; i-immersion line; L-lecithin; S-sphingomyelin; LL-lysolecithin; FA-fatty acids; P-proteins.

the technique described in the Appendix. To avoid the missing of substances in low concentrations, a series with increasing concentration was chromatographed by spotting on the starting points 1 to 6 times. A second chromatogram was made in the same way from the solution in the measurement vessel at the end of the experiment. The resulting chromatograms are shown in Fig. 25 and Fig. 26 respectively.

Comparison of these chromatograms makes it quite obvious that the lysolecithin concentration has greatly increased and the lecithin concentration decreased. In addition, we observe on the chromatogram in Fig. 26 two other spots: one at the starting point representing protein of the added snake venom, and another at the solvent front of the chromatogram originating from the fatty acids split off from the lecithin at the conversion into lysolecithin.

Discussion.

Relating the results of the chromatographic analyses of the solutions before and after the ninety-hour action of the snake venom to the observations and results of the thermo-conductometric measurement, the following points can be noted:

1. Snake venom acts on an opalescent high-resistance lecithin system, converting it into a clear solution.
2. Notwithstanding the association of the lecithin molecules and the presence of 18.2% tertiary butanol, snake venom lecithinase converts the lecithin into lysolecithin and fatty acids.
3. After 40 hours of action the system is labilized because stirring for 2 minutes reduces the resistance factor to lower values (2.32) than in an analogous blank experiment (3.71 in Fig. 23).
4. Compared with the transition temperature of the original system, this temperature is lowered after the action of snake venom.
5. After 90 hours of action the resistance factor curve is situated very low on the graph, much lower even than the curve with constant stirring in Fig. 23.
6. To explain the decrease of the resistance factors, three factors must be considered as possible causes:
   a. The increase in the lysolecithin concentration.
   b. The decrease in the lecithin concentration.
   c. The occurrence of fatty acids in the system.

In the following sections, point 6, sub. a and b will be discussed. Point 6 ,sub. c was not studied, since this would involve a quantitative preparation of the fatty acids splitt off from the lecithin. On theoretical grounds to be discussed later, it must be expected that the presence of fatty acids in the solution will alter the properties of the lecithin. We intend to investigate the influence of saturated and unsaturated fatty acids on lecithin systems in the future.

5. Influence of increasing amounts of lysolecithin on the resistance factor curve.

To differentiate between the influence of increasing concentrations of lysolecithin and decreasing concentrations of lecithin on the resistance factor curve, we studied first the influence of increasing amounts of lysolecithin on a constant amount of lecithin. This experiment also serves to elucidate another problem: whether the small amounts of lysolecithin accompanying lecithin in biological material influence the physico-chemical properties of this lecithin.

Method.

See Chapter IV.

Solutions.

The preparation of lysolecithin from hen's-egg lecithin is described in the Appendix. According to Saunders (1957), the molecular weight of lysolecithin is about 535. To obtain a lysolecithin solution of 8 mmol/L, a stock solution of 4.3% was made up. The following solutions were then prepared:

Table V.

	I ml	II ml	III ml	IV ml	V ml
6.4% lecithin in 91% tert. but.	5.40	5.40	5.40	5.40	5.40
4.3% lysolecithin in 91% tert. but.	—	0.53	1.06	1.59	2.12
Tert. butanol 91%	5.00	4.50	4.00	3.50	3.00
NaCl 20 meq/L	25.00	25.00	25.00	25.00	25.00
Aqua bi-dest.	15.00	15.00	15.00	15.00	15.00
mol. ratio $\frac{\text{lysolec.}}{\text{lecithin}}$	—	0.1	0.2	0.3	0.4

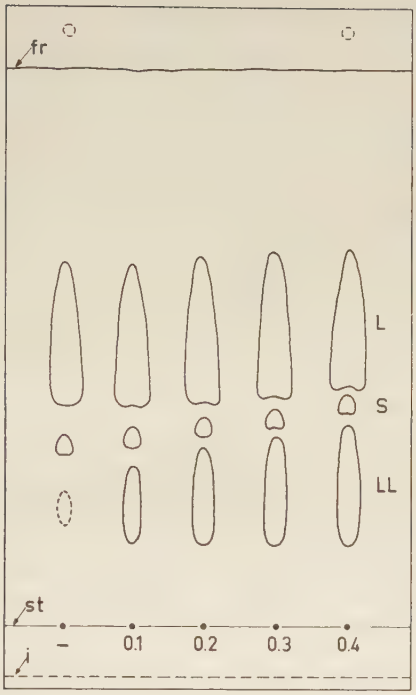


Fig. 27. Chromatogram of solutions of hen's-egg lecithin containing increasing amounts of lysolecithin. The figures below the starting points (on line st) indicate the ratio: lysolecithin – lecithin. L-lecithin; LL-lysolecithin; S-sphingomyelin.

To obtain a visual expression of this composition, the chromatogram shown in Fig. 27 was made on $SiCl_4$-impregnated paper according the technique described in the Appendix. Inspection of the solutions showed that solution V, containing 40% extra lysolecithin, was quite clear at room temperature (20° C), the other solutions being opalescent.

Results.

Plotting the results of the measurements in terms of the relation resistance factor – temperature failed to give a clear picture of the influence of lysolecithin; a more instructive graph was obtained by plotting the isothermic curves expressing the relation, resistance factor – lysolecithin : lecithin ratio.

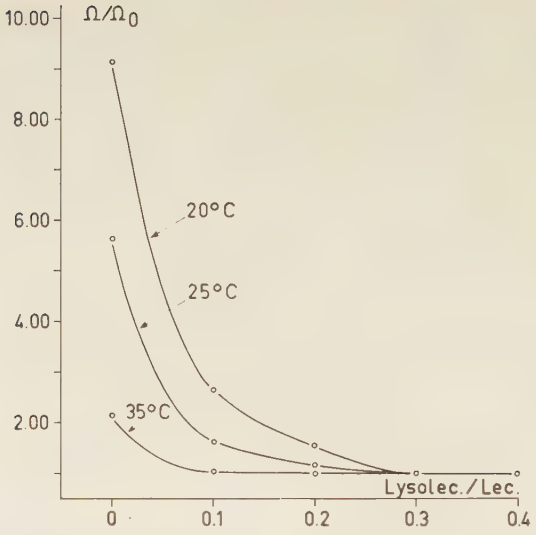


Fig. 28. Influence of increasing amounts of lysolecithin on the resistance factor of constant amounts of lecithin, represented by isothermic curves.

The isothermic curves show clearly that even small additions of lysolecithin counteract the formation of a high resistance system by lecithin. In Figure 28 the isotherm of 20° C reaches the base line (Ω/Ω_0—1.00) at the ratio 0.3; the isotherm at 35° C reaches the base line at the ratio 0.1.

6. **Replacement of lecithin by lysolecithin.**

The preceding section dealt with the influence of lysolecithin on the resistance factor of a constant amount of lecithin. However, the action of snake venom is twofold: an increase of lysolecithin is accompanied by a simultaneous decrease of the lecithin concentration. This effect was studied by maintaining the total phospholipid concentration at 8 mmol/L for different concentrations of lecithin and lysolecithin. A series with decreasing concentration of lecithin was also measured.

Method.

See Chapter IV.

Solutions.

The following solutions were prepared:

Table VI.

	ml	ml	ml	ml	ml	ml	ml
6.4% lecithin in 91 % tert. but.	5.40	4.86	3.78	2.70	1.62	0.54	—
4.3% lysolec. in 91% tert. but.	—	0.53	1.59	2.65	3.71	4.77	5.30
Tert. butanol 91%	5.00	5.00	5.00	5.00	5.00	5.00	5.00
NaCl 20 meq/L	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Aqua bi-dest.	15.00	15.00	15.00	15.00	15.00	15.00	15.00
mmol lecithin/L	8.0	7.2	5.6	4.0	2.4	0.8	0
mmol lysolecithin/L	0	0.8	2.4	4.0	5.6	7.2	8.0

Table VII.

	ml	ml	ml	ml	ml	ml
6.4% lecithin in 91% tert. but.	5.40	4.86	3.78	2.70	1.62	0.54
Tert. butanol 91%	5.00	5.50	6.50	7.50	8.50	9.50
NaCl 20 meq/L	25.00	25.00	25.00	25.00	25.00	25.00
Aqua bi-dest.	15.00	15.00	15.00	15.00	15.00	15.00
mmol lecithin/L	8.0	7.2	5.6	4.0	2.4	0.8

The composition of these solutions was checked by paperchromatography according the technique described in the Appendix. The resulting chromatograms are shown in Fig. 29 and Fig. 30.

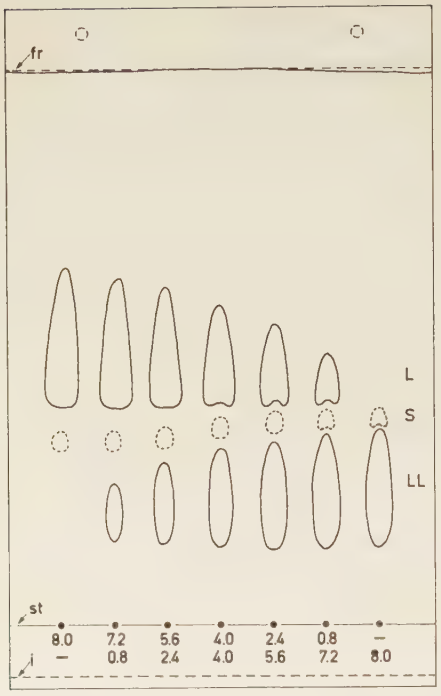


Fig. 29.

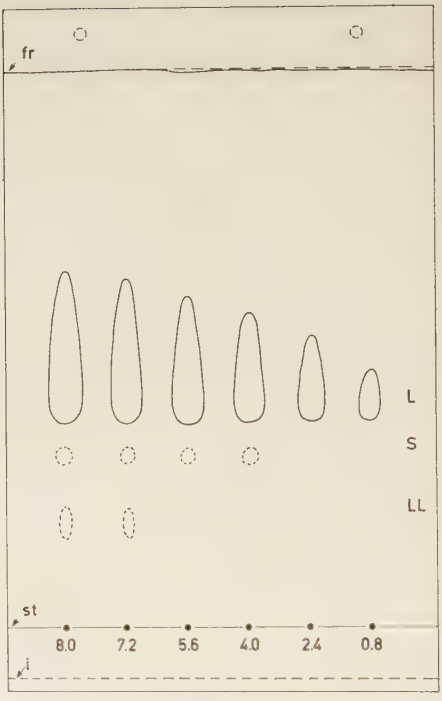


Fig. 30.

Chromatograms on $SiCl_4$-impregnated paper of the series in Table VI and Table VII respectively. The figures below the starting points (on line st) represent mmol Lec./L (in Fig. 30 and upper row in Fig. 29) and mmol Lysolec./L (lower row in Fig. 29). L-lecithin; S-sphingomyelin; LL-lysolecithin.

Results.

During these measurements it was observed that with increasing amounts of lysolecithin the transition point (the temperature at which the opalescent system alters into a clear solution) was lowered.

As in the preceding case, the isothermic relation was found to be the best way to express the results of these measurements graphically. In Fig. 31 we have plotted the isotherms of 20° C for both measured series. Considering the isotherm of lecithin, it is clear that the resulting resistance factor strongly depends on the concentration of this phospholipid.

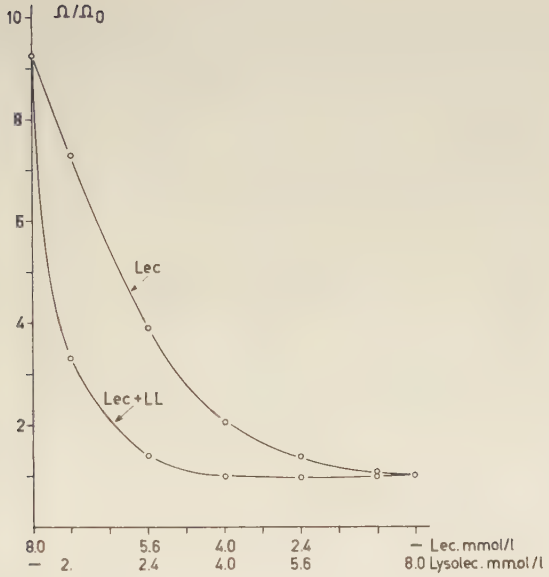


Fig. 31. Isothermic curve (20° C) indicating the effect on the resistance factor when lecithin is replaced by lysolecithin. The total phospholipid concentration is constant in the whole series, namely 8 mmol/L.

These data indicate that there is a linear relation between the square root of the lecithin concentration and the logarithm of the increase of the resistance factor above unit (log $(\Omega—\Omega_0)\Omega_0^{-1}$) which is shown in Fig. 32.

Table VIII.

Data of the lecithin series.

lec. conc. mmol/L	√lec. conc.	Ω 20° C	Ω_0 20° C	Ω/Ω_0	Log $(\Omega-\Omega_0)\Omega_0^{-1}$
8.0	2.83	7500	812	9.236	0.9655
7.2	2.68	5900	812	7.266	0.8613
5.6	2.37	3180	812	3.916	0.4648
4.0	2.00	1680	812	2.069	0.0290
2.4	1.55	1130	812	1.392	0.5933 — 1
0.8	0.89	880	812	1.084	0.9243 — 2

Table IX.

Data of the lecithin-lysolecithin series.

lec. conc. mmol/L	lysolec. conc. mmol/L	$\sqrt{}$lec. conc.	Ω 20° C	Ω_0 20° C	Ω/Ω_0	$\text{Log}(\Omega-\Omega_0)\Omega_0^{-1}$
8.0	—	2.83	7500	812	9.236	0.9655
7.2	0.8	2.68	2695	812	3.319	0.3636
5.6	2.4	2.37	1130	812	1.392	0.5933 — 1
4.0	4.0	2.00	827	812	1.018	0.2553 — 2
2.4	5.6	1.55	812	812	1.000	—
0.8	7.2	0.89	810	812	0.998	—
—	8.0	—	810	812	0.998	—

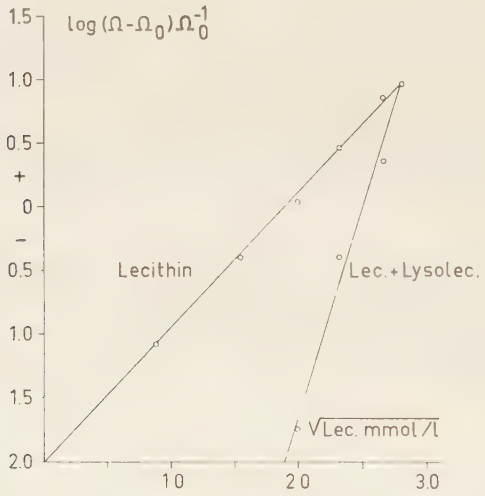


Fig. 32. Relation between the increase of the resistance factor and the lecithin concentration (up to 8 mmol/L), with and without increasing amounts of lysolecithin.

Although the significance of this mathematical relation cannot be understood at this moment in terms of molecular interaction, it is evident from this graph that the gradual substitution of lecithin for lysolecithin alters the physico-chemical properties in a special way which is somehow proportional to the amount of replaced lecithin.

Relating these results to the experiments involving injection of snake venom into a high-resistance lecithin system, we conclude that the decrease in the resistance factor is a reflection of at least two combined effects: an increase in the lysolecithin concentration and decrease in the lecithin concentration. (In this approach we neglect any possible effect from the presence of fatty acids in the solution).

7. Dependence of the resistance factor on the lecithin concentration.

The preceding experiment showed a relationship between the increase of the resistance factor and the lecithin concentration till 8 mmol/L. We then investigated whether the same relationship holds for higher lecithin concentrations.

Method.

See Chapter IV.

Solutions.

The following solutions were used:

Table X.

	ml	ml	ml	ml	ml
6.4% lec. in 91% tert. butanol	2.16	4.32	6.48	8.64	10.80
Tert. butanol 91%	8.00	6.00	4.00	2.00	—
NaCl 20 meq/L	25.00	25.00	25.00	25.00	25.00
Aqua bi-dest.	15.00	15.00	15.00	15.00	15.00
mmol lec./L	3.2	6.4	9.6	12.8	16.0

Results.

In Fig. 33 the dependence of the resistance factor on the lecithin concentration is expressed bij the isothermic curves.
These curves show that at higher lecithin concentrations (above

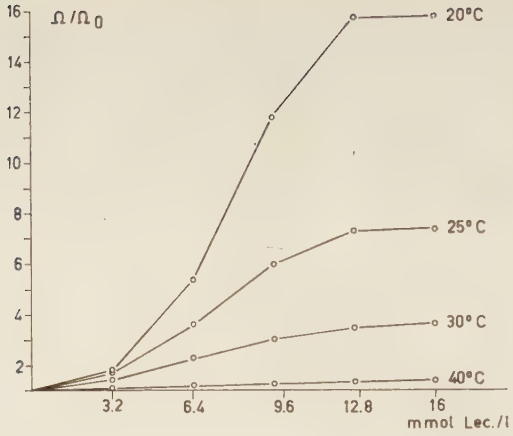


Fig. 33. Dependence of the resistance factor on the lecithin concentration as shown by isothermic curves.

12.8 mmol/L) there is practically no further increase in the resistance factor. This holds particularly for the isotherms at 25° C, and 20° C, which become horizontal at higher lecithin concentrations.

Fig. 34 is a graph showing these results in terms of iso-resistance

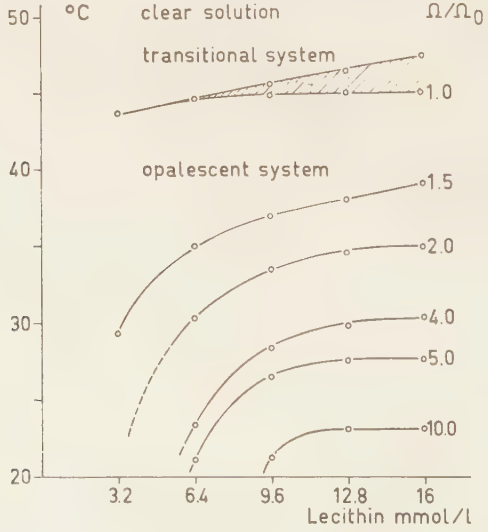


Fig. 34. Iso-resistance factor lines showing the relation of the resisance factor to temperature and lecithin concentration.

factor lines. The course and position of the iso-resistance factor lines appear to be highly dependent on the lecithin concentration, whereas the occurre nce of the opalescence is influenced only slightly by the lecithin concentrations involved in these experiments. At higher lecithin concentrations a higher resistance factor line is reached sooner during a regulated cooling.

The question arises of why there is no further increase in the resistance factor. There may be various reasons for this phenomenon:

1. The arrangement of the lecithin molecules in the solution in membranous structures does not go beyond a certain aggregation state in the solution.
2. The increasing ratio lecithin : tertiary butanol (since the lecithin concentration is increased at a constant tertiary butanol concentration).
3. The increasing ratio lecithin : sodium chloride (since the lecithin concentration is increased at a constant NaCl concentration).

The factors of points 2 and 3 have been made a subject of separate investigation.

8. Dependence of the resistance factor on the tertiary butanol concentration.

In the preceding experiment we altered the lecithin concentration at a constant (18.2%) tertiary butanol concentration. In this experiment we studied the reverse effect, i.e. variations of the tertiary butanol concentration at a constant concentration of lecithin (8 mmol/L). We have already demonstrated (section 1) with the conductometric titration method that lowering the tertiary butanol concentration caused the initial increase in the resistance factor to decrease at a certain tertiary butanol concentration. Although this phenomenon was studied with a purified commercial lecithin preparation and the lecithin concentration decreased during the experiment, it nevertheless illustrates the neccessity for repeating this study with the thermo-conductometric method and with hen's-egg lecithin.

Method.

See Chapter IV.

Solutions.

The following solutions were prepared:

Table XI.

	ml	ml	ml	ml	ml	ml
6.4% lecithin in 91% tert. butanol	5.40	5.40	5.40	5.40	5.40	5.40
Tert. butanol 91%	4.00	4.50	5.00	5.50	6.00	7.00
NaCl 20 meq/L	26.00	25.50	25.00	24.50	24.00	23.00
Aqua bi-dest.	15.00	15.00	15.00	15.00	15.00	15.00
% tert. butanol	16.38	17.29	18.20	19.11	20.02	21.93

To calculate the resistance factors of these opalescent solutions, corresponding solutions were prepared without lecithin and measured in the same way.

Results.

The results of these measurements are shown in Fig. 35 as a temperature – tertiary butanol relation.

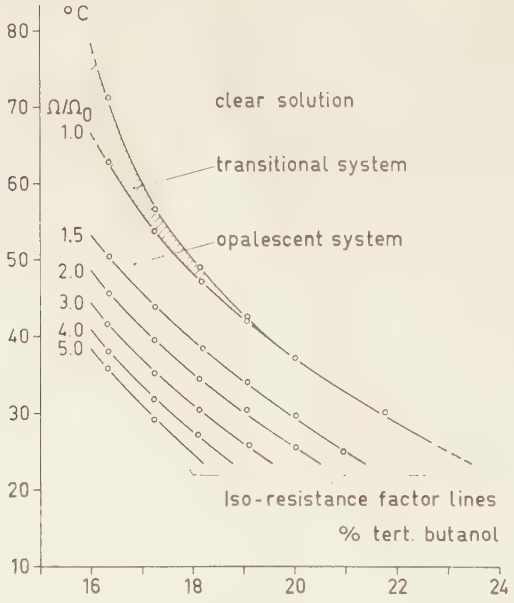


Fig. 35. Iso-resistance factor lines showing the relation of the resistance factor to temperature and tertiary butanol concentration.

The position of the resistance factor lines is seen to be parallel to the lower boundary of the transitional region (line $\Omega/\Omega_0 = 1.0$) which separates the opalescent system from the clear solution.

The resistance factor lines run parallel to each other. This suggests that gradual changes in the tertiary butanol concentration cause only gradual changes in the opalescent system. It must be emphasized that the iso-resistance factor lines reflect only momentary states in this gradual alteration. Extrapolating these results to a pure aqueous solution, very high resistance factors could be expected, but this pure aqueous environment can not yet be realized experimentally.

Discussion.

In the preceding section dealing with the influence of increasing amounts of lecithin on the resistance factor at a constant tertiary butanol concentration, the ratio mmol. lecithin : percentage tertiary butanol varied from 0.18 (3.2 : 18.2) to 0.88 (16 : 18.2) accompanied by a small shift of the transitional point from 43° C to 45° C. In the experiment in this section this ratio was varied from about 0.37 (8 : 21.93) to 0.49 (8 : 16.38) causing a considerable shift of the transitional point from 25° C to 70° C (Fig. 35).

In view of this we must conclude that the increase of the resistance factor at increasing amounts of lecithin (up to a plateau at high concentrations) is not due to alterations in the lecithin : tertiary butanol concentration.

9. Dependance of the resistance factor on the concentration of NaCl.

In the section dealing with the influence of the lecithin concentration on the resistance factor, the question was raised whether the ratio NaCl : lecithin could influence the value of the resistance factor. Since in those experiments this ratio was altered from 10 meq NaCl/3.2 mmol. lecithin to 10 meq NaCl/16 mmol. lecithin. Using the conductometric titration method, Bungenberg de Jong and Lexmond (1957a) found the ultimate resistance factor to be independent of the NaCl concentration to 600 mmol/L.

To study the influence of NaCl, the concentration of this salt was increased while the lecithin concentration was maintained at 8 mmol/L.

Method.

See Chapter IV.

Solutions.

The following solutions were prepared:

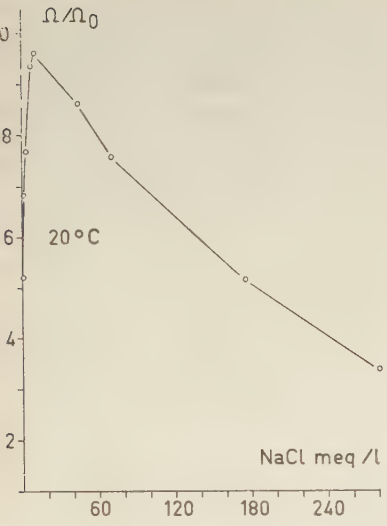
Table XII

ml 6.4% lec. in 91% tert. but.	ml tert. but. 91%	ml NaCl 20 meq/L	ml NaCl 350 meq/L	ml H_2O	NaCl conc. meq/L
5.4	5.0	1.0	—	39.0	0.4
5.4	5.0	2.5	—	37.5	1.0
5.4	5.0	5.0	—	35.0	2.0
5.4	5.0	16.0	—	24.0	6.4
5.4	5.0	25.0	—	15.0	10.0
5.4	5.0	—	6.0	34.0	42.0
5.4	5.0	—	10.0	30.0	70.0
5.4	5.0	—	25.0	15.0	175.0
5.4	5.0	—	40.0	—	280.0

Blank solutions containing 5.0 ml 91% tertiary butanol instead of 5.4 ml of the lecithin stock solution were also prepared and measured.

Results.

After calculation of the ultimate resistance factors (at 20° C) of the different solutions, these values were plotted as a function of the NaCl concentration. The resulting isothermic curve is given in Fig. 36; Fig. 37 shows the part of the isotherm at lower salt concentrations on a larger scale.



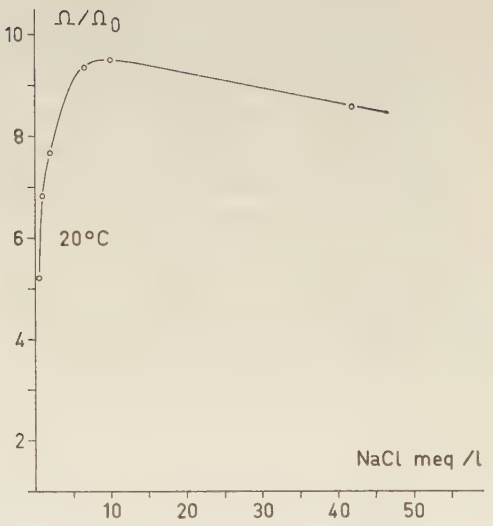


Fig. 36 and 37. Influence of NaCl on the resistance factor (at 20° C) of a solution containing 8 mmol Lec./L. Fig. 37 with the abscissa on a larger scale.

Discussion.

The resistance factor is found to depend strongly on the sodium chloride concentration. Initially, the resistance factor increases very rapidly, followed by a more gradual decrease after a maximum at about 10 meq NaCl/L. Thus, the question raised in section 7, i.e. whether alterations in the lecithin/NaCl ratio cause higher resistance factors, must be answered affirmatively. However, the range of the NaCl concentration in section 7 lies between 5 and 40 meq NaCl/L, calculated for 8 mmol lec./L and in Fig. 37 we observe that in this range the salt influence on the resistance factor is 0.5 maximally. The results of the experiments in section 7 thus cannot be explained bij alterations in the ratio mmol. lecithin : meq NaCl.

10. Dependence of the resistance factor on the $CaCl_2$ concentration.

Leathes (1925), describing his microscopical observations on the formation of myelinic figures in a lecithin preparation, already ment-

ioned the inhibition of this formation by Ca ions. It has been shown in the preceding section that the capacity of lecithin to increase the resistance factor is largely suppressed by large concentrations of NaCl. We therefore wondered whether the presence of $CaCl_2$ would have an analogous or even a much stronger effect, since calcium cations show a much higher affinity for the negative phosphate group than sodium cations.

Method.

See Chapter IV.

Solutions.

Table XIII shows the lecithin solutions containing various amounts of $CaCl_2$. The blanks were prepared in the same way.

Table XIII.

ml 6.4% lec. in 91% tert. but.	ml tert. but. 91%	ml $CaCl_2$ 36 meq/L	ml $CaCl_2$ 720 meq/L	ml H_2O	$CaCl_2$ conc. meq/L
5.4	5.0	1.0	—	39.0	0.72
5.4	5.0	4.0	—	36.0	2.88
5.4	5.0	20.0	—	20.0	14.40
5.4	5.0	—	5.0	35.0	72.20
5.4	5.0	—	10.0	30.0	144.00
5.4	5.0	—	20.0	30.0	288.00
5.4	5.0	—	40.0	—	576.00

Results.

The resistance factors at 20° C are plotted in Fig. 38 in the same way as was done for the experiments with NaCl.

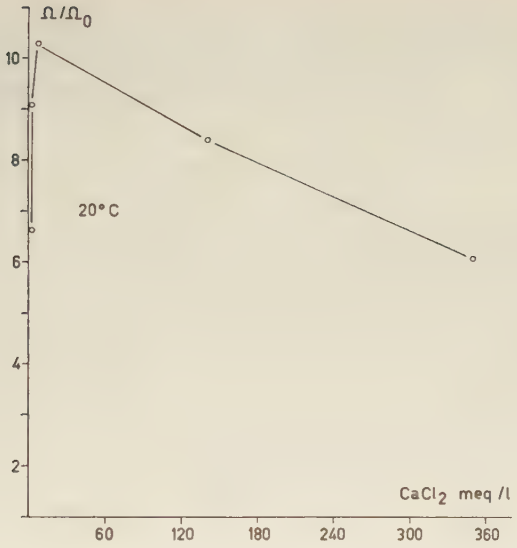


Fig. 38. Influence of $CaCl_2$ on the value of the resistance factor at 20° C.

The isotherm in Fig. 38 shows a high degree of correspondence with that in Fig. 36 showing the influence of NaCl, but the $CaCl_2$-isotherm is situated over its whole length at higher resistance factor values. The initial effect is somewhat stronger here, but there is less disturbing effect at higher $CaCl_2$ concentrations.

11. **Dependence of the resistance factor on the pH of the medium.**

The preceding experiments with egg lecithin were performed at an arbitrary pH of about 6 ,this being the pH of the combined solutions of the systems to be measured. Since the hydrophilic part of the lecithin molecules carries a negative and a positive group, the lecithin molecule will behave as an amphoion. On the basis of the dissociation constants of the phosphoric acid group and the choline group, the iso-electric point of lecithin was calculated by Fischgold and Chain (1934) to be 7.5. In the next experiments we studied the effect of variations in the pH of the medium on the resistence factor.

Method.

See Chapter IV. For this investigation we used the small measurement vessel II.

Solutions.

The various pH values were reached by increasing the amount of NaOH 0.009 N added to the lecithin system (constant final volume 10 ml) containing a constant amount of HCl 0.01 N (2.0 ml). The amount of NaCl thus formed in the solutions ranged from 0 to 4 meq/L (at pH 7.0). However, in section 9 it was seen that the presence of even such small amounts of NaCl greatly influences the behaviour of the lecithin. In this range (up to about 5 meq/L) the resistance factor rises steeply; from 6 to 13 meq NaCl/L the effect was very slight (see Fig. 37). To minimize the contribution of the salt effect to the end results of the measurement, we limited the variations of NaCl concentration between 6 and 13 meq/L by supplying each system with an additional amount of 6 meq NaCl/L (excepting the last system). According to these data systems with various pH values as indicated in Table XIV were prepared.

Table XIV.

no.	ml 3.2% lec. in 91% tert. but.	ml HCl 0.01 N	ml NaOH 0.009 N	ml NaCl 30 meq/L	ml H_2O	pH before	pH after
1	2.0	6.0	—	2.0	—	2.38	2.42
2	2.0	2.0	—	2.0	4.0	2.91	2.94
3	2.0	2.0	1.0	2.0	3.0	3.26	3.31
4	2.0	2.0	2.0	2.0	2.0	5.80	5.98
5	2.0	2.0	2.2	2.0	1.8	7.01	7.05
6	2.0	2.0	2.4	2.0	1.6	8.65	7.18
7	2.0	2.0	3.0	2.0	1.0	9.95	7.56
8	2.0	2.0	3.4	2.0	0.6	10.49	8.25
9	2.0	2.0	4.0	2.0	—	10.73	10.03
10	2.0	2.0	8.0	2.0	—	11.61	11.59

These systems were not prepared at the same time; each one was made up separately just before a thermo-conductometric measurement. After filling the small measurement vessel, the rest of the solution was used for the measurement of the pH. The blank systems were similarly prepared but contained 91% tertiary butanol instead of the lecithin solution.

To obtain a preliminary impression of the influence of the hydrogen ion concentration, we first measured the systems at pH 7.01 and pH 10.73, and the corresponding blank systems.

Results.

Fig. 39 gives the resistance factor curves of systems at pH 7.01 and pH 10.73. This graph shows clearly that alteration of the hydrogen ion concentration has a very strong effect on the resistance factor curve. At the end of these measurements the pH of the heated and cooled systems were measured again to check their stability. The pH values were 7.05 and 10.03 respectively, the latter having decreased by 0.70. The other systems mentioned in Table XIV were then measured thermo-conductometrically as well as the correspond-

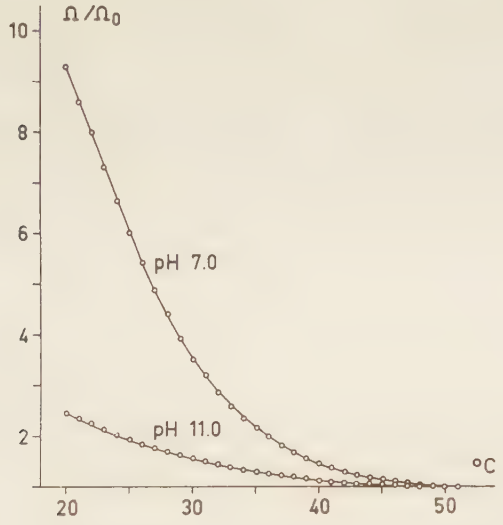


Fig. 39. Influence of the pH of the medium on the resistance factor curve of a system containing 8 mmol lecithin/L in 18.2% tertiary butanol.

ing blank systems. The pH of the systems was checked before and after measurement; these values are given in the last two columns of the Table. Plotting these values against the concentrations of added NaOH, we obtain the titration curve shown in Fig. 40.

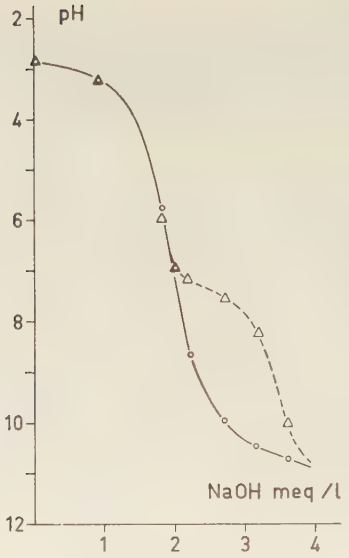


Fig. 40. Titration curve of a lecithin solution (previously acidified with 2 ml HCl 0.01 N) with NaOH. The pH values before and after thermo-conductometric measurement are indicated by circles and triangles respectively.

The circles indicate the pH values of the freshly-prepared solutions; the triangles represent the pH values read after the thermo-conductometric measurement. The resistance factur curves were then

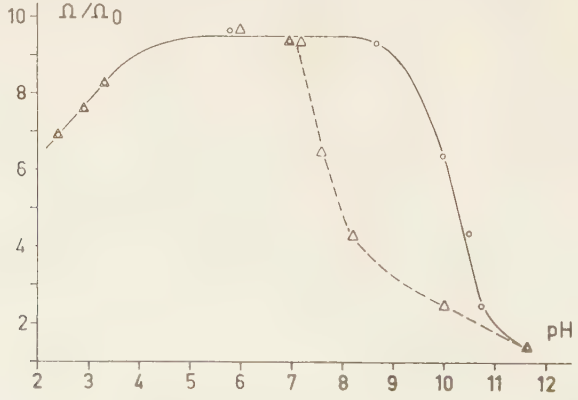


Fig. 41. Isothermic curve (20° C) showing the resistance factor as a function of the pH of the medium. The circles and triangles indicate the pH values before and after the thermo-conductometric measurement respectively.

calculated. Fig. 41 is based on the ultimate resistance factors at 20° C only. With the introduction of both pH values into this Figure (i.e. before (circles) and after measurement (triangles)) the isothermic curve splits off at about pH 7.

The isotherm in Fig. 41 shows a horizontal maximum in the middle range from pH 5 to pH 8.5. Above and below these values the curve slopes downwards. Since the ionic group of lecithin consists of choline as a strong base and phosphoric acid as a strong acid, it will be evident that the I.E.P. of lecithin (about pH 7) is merely an iso-electric range. Relating this range to the horizontal part of the isotherm in Fig. 41 we must conclude that the iso-electric state of the lecithin molecules is the most favourable one for association, giving rise to a high resistance factor. From this point of view gradual decompensation of one of the charged groups, leading to a surplus charge (either negative or positive) of the lecithin molecules. This surplus charge counteracts a very close association of the lecithin molecules, which leads to a relatively lower resistance factor. However, above pH 7 it seems unlikely that the line as drawn represents the real relationship between pH and resistance factor, since the former decreases in the course of the measurement. We have no proof that we may combine the initial pH with the final resistance factor.

By relating the resulting resistance factor to the terminal pH of the system, we obtain the dotted isothermic curve drawn in Fig. 41. If the original pH had been preserved, the lecithin systems might have shown higher resistance factors(up to the horizontal level).

The change in the pH is probably related to other changes in the solutions which also interfere with the properties of the lecithin micellar system. This can be deduced from the following observations.

The pH of system N°6 (Table XIV) changes from 8.65 to 7.18. Notwithstanding this alteration, the ultimate resistance factor coincides fairly well with the horizontal level of the curve in Fig. 41. The pH of system N°7 changes from 9.95 to 7.56. The ultimate pH value thus lies between the pH values of N°6. We could therefore expect that the ultimate resistance factor of system N°7 would also coincides with the horizontal part of the curve, but this resistance factor is much lower ($\Omega/\Omega_0 = 6.7$).

Two combined effects are probably involved here:

a. A decrease in the resistance factor due to a downward shift of the pH.

b. A decrease in the resistance factor more or less independent of any change in pH, probably due to the formation of lysolecithin.

To examine changes in the lecithin system during heating and cooling, the systems were chromatographed according the technique described in the Appendix. It was found that changes in the pH of the systems coincide with the appearance of green spots, belonging to fatty acids, at the front line of the chromatogram. The area of the lecithin spot decreases and that of the lysolecithin increases. Since these chromatographic variations were most pronounced at pH 11.61, a comparable chromatogram is given in Fig. 42 in which

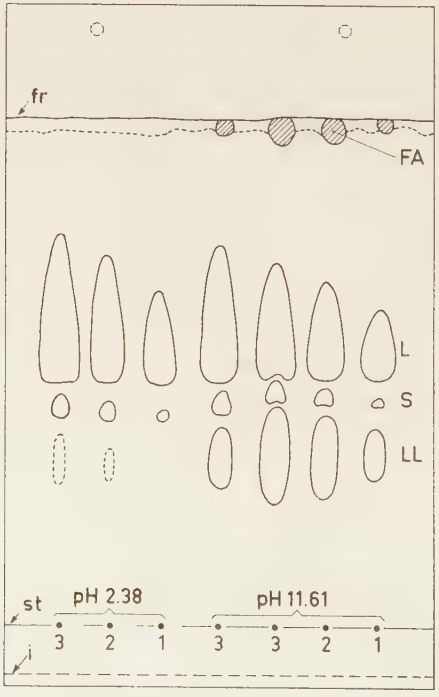


Fig. 42. Chromatogram showing the alterations in a lecithin system at different pH values during a thermo-conductometric measurement. FA-fatty acids; L-lecithin; S-sphingomyelin; LL-lysolecithin.

the results of sampling systems N⁰ 1 and N⁰ 10 are shown. On the three lefthand starting points, system N⁰ 1 (pH 2.38) was spotted 3, 2, and 1 times from left to right. On the three starting points to the right, system N⁰ 10 (pH 11.61) was spotted from right to left 1, 2, and 3 times (after heating). On the starting point in the middle, a sample of the remainder of system N⁰ 10 was brought up after standing for a period equivalent to the heating course at room temperature (20° C). The alterations in the system evidently result from the splitting off of fatty acids from the lecithin molecules and a consequent formation of lysolecithin.

Conclusion.

It has been found that the pH of the medium greatly influences the resistance factor value of the lecithin system. Besides the pH-effect, another phenomenon can be observed above the iso-electric point of lecithin. Above pH 7, part of the lecithin is converted during the thermo-conductometric measurement into lysolecithin due to the splitting-off of one fatty acid. These fatty acids cause a decrease in pH. Lysolecithin acts as a strong lytic agent on the structures formed by the lecithin molecules, causing a sharp decrease in the resistance factor (see section 6).

12. Influence of cholesterol on the system lecithin – 18.2% tertiary butanol in the presence of $CaCl_2$ (280 mmol/L).

In biological material the phospholipids are often present together with cholesterol and cholesterol esters. Cholesterol molecules are probably present in the cell membranes of many types of cells. It is of some interest to know whether cholesterol addition has any effect on the resistance factor measured in our in vitro experiments.

Investigations by Bungenberg de Jong and Davids (1957a) have shown that cholesterol shifts the transitional point of a lecithin system (obtained from a rather impure lecithin (Merck)) to inconveniently high temperatures. As we have seen, the presence of relatively high salt concentrations shifts the transitional point to lower temperatures (section 10); this facilitates the experimental study of the effect of cholesterol on lecithin systems.

Method.

See Chapter IV.

Solutions.

For this experiment 300 ml of each of two stock solutions were prepared. Besides 18.2% tertiary butanol and 280 mmol $CaCl_2$/L, one of these solutions contained lecithin in a concentration of 0.64%. From these stock solutions, 50 ml was added by pipette to duplicate weighed samples of cholesterol. The weighed amounts of cholesterol were chosen such that in the solutions containing lecithin, the molecular ratio cholesterol – lecithin increased in the following way: 1 : 16; 1 : 8; 1 : 4; 1 : 3; 1 : 2.

Observations.

1. The turbidity of the prepared systems increased with increasing amounts of cholesterol.
2. The transitional point of the original lecithin system is modified into a transitional region situated at ever higher temperatures with increasing amounts of cholesterol, as follows:

Table XV.

ratio chol. : lecithin	transitional temperature ° C.
—	34.5
1 : 16	37.1 - 37.5
1 : 8	39.1 - 40.5
1 : 4	42.2 - 45.9
1 : 3	43.6 - 49.6
1 : 2	46.2 - ?

3. Under regulated cooling, at higher cholesterol lecithin ratios (1 : 3 and 1 : 2) the opalescent system shows a more turbid character than at the lower ratios. This turbidity was very marked at lower temperatures.

4. Under cooling of the blank systems it was found that cholesterol, dissolved at higher temperatures, crystallized out at decreasing temperatures.

Results.

The data from Table XV are plotted in Fig. 43; the iso-resistance factor lines are also given. This graph shows a gradual shift of the transitional region to higher temperatures. This upward trend is not

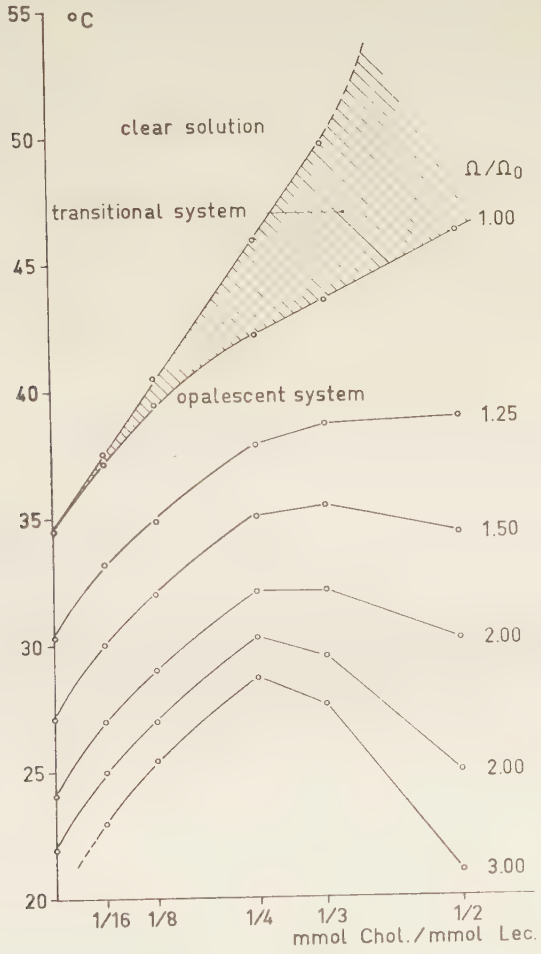


Fig. 43. Influence of cholesterol on the transitional region and the iso-resistance factor lines of a lecithin system containing 280 mmol $CaCl_2$/L.

shown by the iso-resistance factor lines at higher cholesterol concentrations. Evidently there is an optimal ratio of cholesterol to lecithin, giving a maximal resistance factor value.
By plotting the results of the measurements as an isothermic relation, we obtain Fig. 44.

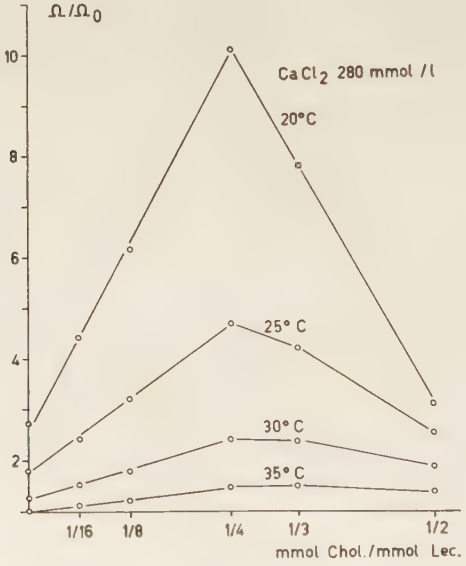


Fig. 44. Isothermic curves showing the relation between the resistance factor and the ratio of cholesterol to lecithin in a system containing 280 mmol $CaCl_2$/L.

When the cholesterol : lecithin ratio is increased, a practically linear relationship is found between the resistance factors and these ratios up to a ratio of 1 : 4. The isotherm at 20° C shows a pronounced maximum at this ratio. At higher temperatures this maximum is less pronounced and even alters into a maximum range from 1 : 4 to 1 : 3 at 35° C.

Discussion.

It has been found that cholesterol can fully restore the original high resistance factor of a lecithin system which is initially reduced by the presence of 280 mmol $CaCl_2$/L. It appeared that the lecithin

system can take up relatively high amounts of cholesterol, but this property is greatly reduced at lower temperatures of the system. A distinct optimal ratio is found at 20° C.

Leathes estimated the cross-sectional areas of lecithin and fatty acids as respectively 56.6 $Å^2$ and 21 $Å^2$. Considering the cross-sectional area of cholesterol to be 40.8 $Å^2$, we would expect an optimal molar ratio of 1 cholesterol : 3 lecithin, calculated on the basis of the available space between the carbon chains of the lecithin molecules. However, Van Deenen and co-workers (1962) reported the great influence of the character of the fatty acids present on the cross-sectional area of the lecithin molecules in spread monolayers. Any calculation without exact data on the fatty acid composition of the lecithin involved is therefore of no value.

The cross-sectional area of the molecules in a spread monolayer increases with higher temperatures. Our experiments suggest that at higher temperatures more cholesterol molecules combine with lecithine in a complex association. When the temperature is lowered the excess cholesterol crystallizes out just as in the blank systems; this disturbs the association, as indicated by a lowering of the resistance factor.

13. Comparison of the effect of cholesterol and lysolecithin on the resistance factor curve of lecithin systems.

To close the experiments with egg lecithin, we made comparative measurements of two different lecithin preparations, with and without addition of cholesterol or lysolecithin in the presence of 10 meq NaCl/L. Both compounds are generally present together with lecithin in biological material. Our studies show the great variability of the physico-chemical properties of lecithin systems caused by the presence of these substances.

Method.

See Chapter IV.

Solutions.

For these measurements we used fresh lecithin preparations made from the yolks of hen's eggs (lecithin I and lecithin II). The following systems (1-8) were prepared. See Table XVI.

Table XVI.

	1	2	3	4
ml 6.4% lec. I in 91% tert. but.	5.38	—	5.38	—
ml 91% tertiary butanol	5.00	10.00	—	5.00
ml 0.77% chol. in 91% tert. but	—	—	5.00	5.00
ml NaCl 20 meq/L	25.00	25.00	25.00	25.00
ml aqua bi-dest.	15.00	15.00	15.00	15.00
	5	6	7	8
ml. 6.4% lec. II in 91% tert. but	5.38	—	5.38	—
ml 91% tertiary butanol	5.00	10.00	4.50	9.50
ml 4.3% lysolecithin in 91% tert. but.	—	—	0.54	0.54
ml NaCl 20 meq/L	25.00	25.00	25.00	25.00
ml aqua bi-dest.	15.00	15.00	15.00	15.00

It will be clear from this Table that the solutions with the even numbers 2, 4, 6, and 8 are blank systems of the directly preceding uneven numbers.

Results.

After calculation, the resistance factors were plotted against their temperatures. The four resulting resistance factor curves are shown in Fig. 45. The standard curves of both preparations differ only slightly in the temperature range from 24° C to 20° C (curves lec. I and lec. II in the graph). The addition of cholesterol to the lecithin system (ratio 1 : 4) in the presence of 10 meq NaCl/L gives a very high increase in the resistance factor ($\Omega/\Omega_0 = 21.0$ at 20° C), whereas the addition of lysolecithin (ratio 1: 10) resulted in a decrease in this factor to 2.25. This graph expresses the strong effect of these biologically important substances on the physico-chemical properties of a lecithin system.

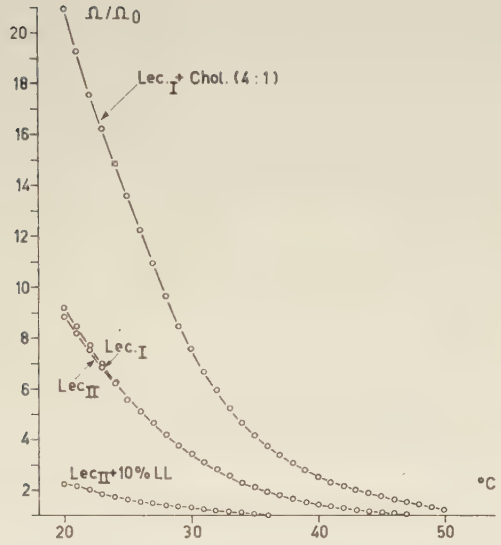


Fig. 45. Resistance factor curves of different lecithin preparations (Lec. I and Lec. II) before and after addition of cholesterol or lysolecithin.

II. EXPERIMENTS WITH BEEF SPINAL-CORD LECITHIN.

Introduction.

The preceding experiments with lecithin prepared from the yolk of hen's eggs elucidated some of the physico-chemical properties of this lecithin preparation, but it could not be concluded that lecithins from other sources would show the same phenomena under the same experimental circumstances. Egg-yolk lecithin might be considered a rather special type of lecithin. Results obtained with egg lecithin therefore might not be representative for lecithin in biological membranes generally.

A quite different source of phospholipids is the central nervous system. It has long been known that the white matter contains large amounts of lipids. Electron-microscopy has located these lipids in circular membranes around the axons. Geren (1954) found that these membranes originate from the multifolded cell membranes of the Schwann cells. Using beef spinal cord as a source of lecithin

provides a preparation whose individual molecules were previously bound in membrane structures. The results of the study of this spinal-cord lecithin preparation can be used to determine whether the data provided by the investigation of egg lecithin have a more general validity or are limited to that particular lecithin.

We first observed the optical phenomena shown by this preparation in the presence of different amounts of tertiary butanol.

1. The temperature – tertiary butanol diagram.

Method.

The method and equipment are described in section 1 dealing with the optical study of hen's-egg lecithin.

Solutions.

The various systems were prepared using a stock solution of 6.4% lecithin in 91% tertiary butanol (see Table XVII). Anticipating the termo-conductometric measurements, these solutions also contained NaCl.

Table XVII.

	ml	ml	ml	ml	ml	ml	ml
6.4% lecithin in 91% tert. but.	0.54	0.54	0.54	0.54	0.54	0.54	0.54
Tertiary butanol 91%	0.60	0.65	0.70	0.75	0.80	0.85	0.90
NaCl 20 meq/L	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Aqua bi-dest.	1.40	1.35	1.30	1.25	1.20	1.15	1.10
% tert. butanol	20.02	20.93	21.84	22.75	23.66	24.57	25.48

Results.

Table XVIII gives the mean values of the temperatures at which the optical changes were observed.

Table XVIII.

% tert. butanol	transitional temp. ° C.
20.02	57.3 - > 80
20.93	49.3 - > 80
21.84	43.4 - 51.5
22.75	38.7 - 42.4
23.66	34.5 - 36.9
24.57	30.9 - 32.0
25.48	27.4

The values in this Table are plotted in Fig. 46. The resulting graph shows a close resemblance to that found for egg lecithin (Fig. 21), except that the phenomena are shifted to higher tertiary butanol concentrations. The observed optical phenomena and the position of the different systems suggested that the properties of this lecithin could be studied with the termo-conductometric technique.

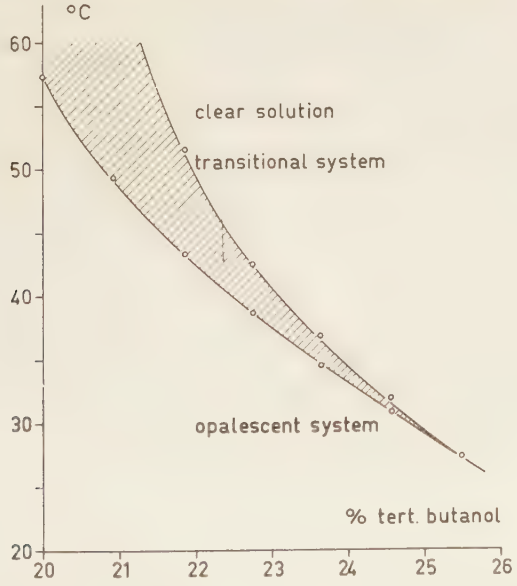


Fig. 46. Temperature – tertiary butanol diagram of spinal-cord lecithin in the presence of 10 meq NaCl/L.

2. Influence of the tertiary butanol concentration on the resistance factor.

From the results of the preceding section dealing with the optical phenomena of spinal-cord lecithin, a tertiary butanol concentration can be selected at which the lecithin system can be converted into a clear system at a convenient temperature with maintenance of a broad opalescent region. However, such selection assumes an identity between this opalescent region and that of egg lecithin, and this had previously determined by thermo-conductometric measurements.

Method.

See Chapter IV.

Solutions.

The following solutions were prepared:

Table IXX.

	I ml	II ml	III ml	IV ml	V ml	VI ml
6.4 % lecithin in 91% tert. but.	5.40	5.40	5.40	5.40	5.40	5.40
Tertiary butanol 91%	6.00	7.00	7.50	8.00	8.50	9.00
NaCl 20 meq/L	25.00	25.00	25.00	25.00	25.00	25.00
Aqua bi-dest.	14.00	13.00	12.50	12.00	11.50	11.00
% tert. butanol	20.02	21.84	22.75	23.66	24.57	25.48

To evaluate the results in terms of the resistance factors, analogous blank solutions were prepared by adding an amount of 91% tertiary butanol corresponding to the amount of solvent in the lecithin stock solution.

Results.

After calculation of the resistance factors of each temperature course, data were derived from them to plot the iso-resistance factor lines as shown in Fig. 47.

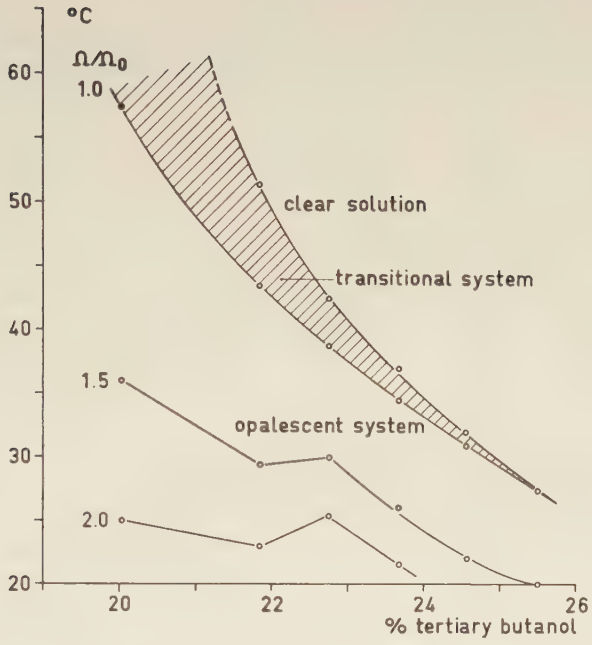


Fig. 47. Iso-resistance factor lines showing the relation between temperature and tertiary butanol concentration of a system containing spinal-cord lecithin.

Comparing the graph in Fig. 47 with the corresponding graph for egg lecithin given in Fig. 35, we observe that the transitional system of the spinal-cord lecithin is broader and situated at higher tertiary butanol concentrations. This suggests that spinal-cord lecithin is less soluble in H_2O – tertiary butanol mixtures than egg lecithin. In view of the course of the basic resistance factor line ($\Omega/\Omega_0 = 1.0$), we had expected to find corresponding parallel iso-resistance factor lines, but Fig. 47 shows a good deal of irregularity compared with the corresponding lines in Fig. 35. This irregularity is more clearly expressed when the results are plotted as iso-thermic curves, as given in Fig. 48. The initial steep rise of these curves is suddenly altered into a downward slope at a tertiary butanol concentration of 22.75%. This graph closely resembles that found for the iso-thermic relation of the resistance factor and addition of cholesterol to a system of egg lecithin (Fig. 44). At higher cholesterol concen-

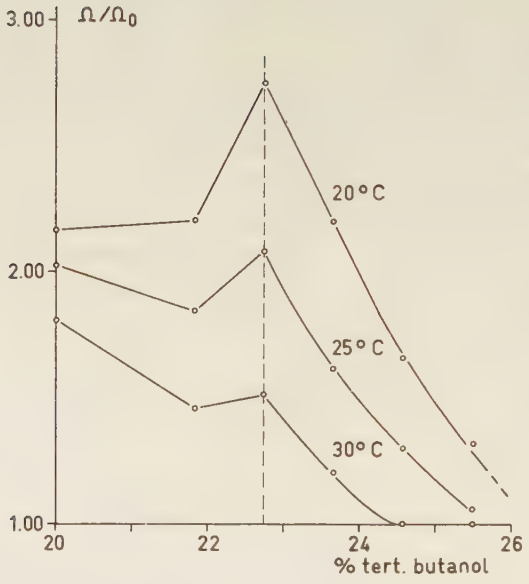


Fig. 48. Isothermic curves relating the concentration of tertiary butanol to the resistance factors of a spinal-cord lecithin system.

trations, the initial amount of cholesterol taken up at higher temperatures by the egg lecithin crystallizes out at lower temperatures, causing a strong decrease in the resistance factor. This resemblance suggests that in the case of spinal-cord lecithin crystallization is also a limiting factor for a further increase of the resistance factor under cooling in the presence of lower tertiary butanol concentrations.

From Fig. 48 we conclude that the optimal tertiary butanol concentration for further investigation of spinal-cord lecithin is 22.75%. At this concentration the resistance factor can increase by a factor of 1.68 maximally.

3. **Influence of the $CaCl_2$ concentration on the resistance factor.**

These experiments were performed in the presence of 22.75% tertiary butanol.

Method.

See Chapter IV.

Solutions.

The following solutions were prepared:

Table XX.

	ml	ml	ml	ml	ml
6.4% lecithin in 91% tert. butanol	5.40	5.40	5.40	5.40	5.40
Tert. butanol 91%	7.50	7.50	7.50	7.50	7.50
$CaCl_2$ 700 meq/L	1.00	5.00	15.00	25.00	37.50
Aqua bi-dest.	36.50	32.50	22.50	12.50	—
meq $CaCl_2$/L	14	70	210	350	525

Blanks were again prepared in order to express the results in terms of the resistance factor.

Results.

Increasing the amount of $CaCl_2$ in the solutions showed that the addition of 14 meq $CaCl_2$/L shifted the transitional region about 3° C upwards as compared to the position of a corresponding system containing 10 meq NaCl/L. This initial upward shift was also found for egg lecithin. At higher $CaCl_2$ concentrations, however, a gradual lowering of the transitional temperature was found.

By plotting the isotherms at 20°, 25°, and 30° C, the influence of the $CaCl_2$ on the resistance factor is clearly shown in Fig. 49. The isotherm at 20° C shows a pronounced upward course at relatively small concentrations of $CaCl_2$, whereas at higher concentrations we found lower values of the resistance factor. The shape of the curve closely resembles Fig. 38 (influence of $CaCl_2$ on egg lecithin), although the whole curve is situated at lower resistance factors.

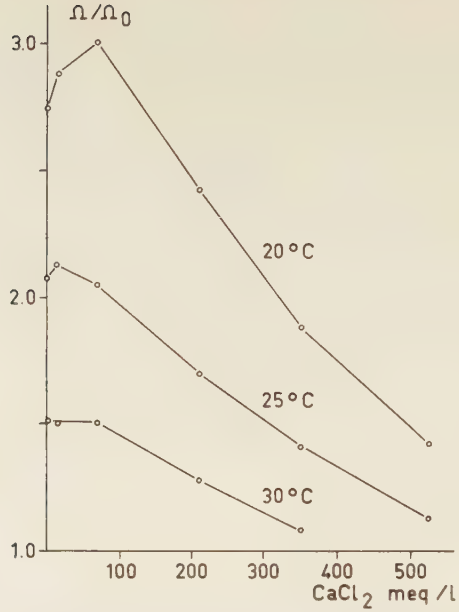


Fig. 49. Influence of the $CaCl_2$ concentration on the resistance factor of a spinal-cord lecithin system, presented by the isotherms at 20°, 25° ,and 30° C.

4. Influence of cholesterol additions on the resistance factor of a system containing calcium ions.

To permit further comparison of the physico-chemical behaviour of spinal-cord lecithin with that of egg yolk lecithin, we made a study of the influence of cholesterol additions on the resistance factor. This study was performed on a spinal-cord lecithin system in which the resistance factor values were reduced by the addition of 525 meq $CaCl_2$/L.

Method.

See Chapter IV.

Solutions.

To realize a series with increasing ratios of cholesterol : lecithin, increasing amounts of cholesterol were added to a constant amount

of lecithin. This was done by pipetting various amounts of a cholesterol stock solution containing 66.67 mmol cholesterol/L (674.5 mg cholesterol dissolved in 91% tertiary butanol upto 25 ml) to the lecithin solutions as shown in Table XXI.

Table XXI.

	ml	ml	ml	ml	m'
6.4% lecithin in 91% tert. butanol	5.40	5.40	5.40	5.40	5.40
Chol. 66.67 mmol/L in 91% tert. but.	—	0.60	1.20	1.80	2.40
Tert. butanol 91%	7.50	6.90	6.30	5.70	5.10
$CaCl_2$ 700 meq/L	37.50	37.50	37.50	37.50	37.50
mmol Chol. : mmol Lec.	—	1 : 10	2 : 10	3 : 10	4 ; 10

Results.

These solutions, and the corresponding solutions without lecithin, were used in experiments to determine resistance factors. The measurements showed that with increasing cholesterol concentration the position of the transitional region was shifted to higher temperatures, coupled with a broadening of this region. The results are given in Fig. 50 in the form of the isothermic relation between the resistance factor and the cholesterol-lecithin ratio.

This graph shows generally an increase in the resistance factor with increasing amounts of cholesterol, the effect being most pronounced at lower temperatures. The isotherm for 20° C suggests an optimal ratio at about 2.8, corresponding with 1 mmol lecithin : 3.6 mmol cholesterol.

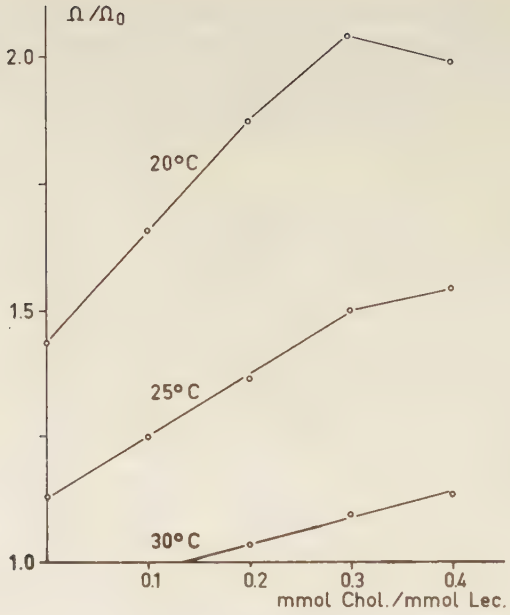


Fig. 50. Isothermic curves representing the relation between the resistance factor and the cholesterol : lecithin ratio of a system containing 525 meq $CaCl_2$/L.

5. Comparison of the results obtained with the thermo-conductometric measurements of beef spinal-cord lecithin and hen's egg-yolk lecithin.

In view of the following phenomena shown by both lecithin preparations, we must conclude that their physico-chemical behaviour shows remarkable analogies:

1. A corresponding optical phenomenon on cooling of a preheated system : clear solution, transitional system, and opalescent system.
2. The appearance of the opalescent system coincides with an increase in the resistance factor.
3. Systems containing added cholesterol show, with both lecithins, an optimal increase in the resistance factor around the molar ratio 1 : 4 (cholesterol : lecithin), at 20° C.

4. With both lecithins the optimal cholesterol : lecithin ratio increased with increased temperatures of the system.
5. Increase of the $CaCl_2$ concentration in the solution resulted in reduction of the resistance factor to very low values after an initial increase.

However, we found the following differences:

1. To investigate the points mentioned above, the concentration of tertiary butanol had to be raised for spinal-cord lecithin to 22.75% (for egg lecithin 18.2%).
2. The ultimate resistance factor is lower for spinal-cord lecithin than for egg lecithin.
3. Egg lecithin in the presence of 22.75% tertiary butanol (as used with spinal-cord lecithin) shows a lower ultimate resistance factor than spinal-cord lecithin.
4. The iso-resistance factor lines of spinal-cord lecithin show an irregular course above 22.75% tertiary butanol.
5. The transitional region for spinal-cord lecithin is broader than that for egg lecithin.

III. EXPERIMENTS WITH BEEF SPINAL-CORD SPHINGOMYELIN.

Besides lecithin, other phospholipids can be prepared from beef spinal cord in relatively large amounts, particularly sphingomyelin and cephalin. The question arises whether sphingomyelin would show phenomena analogous to those observed in the lecithin preparation.

1. Temperature – tertiary butanol diagrams.

a. Temperature – tertiary butanol diagram in the presence of 10 meq NaCl/L.

Method.

See Chapter IV.

Solutions.

The following solutions were prepared:

Table XXII.

	ml	ml	ml	ml	ml	ml	ml	ml	ml	ml
6.4% sphingomyelin in 91% tert. but.	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54
Tert. butanol 91%	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.90	1.00	1.15
NaCl 20 meq/L	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Aqua bi-dest.	1.50	1.45	1.40	1.35	1.30	1.25	1.20	1.10	1.00	0.85
% tertiary butanol	18.20	19.11	20.02	20.93	21.84	22.75	23.66	25.48	27.30	30.03

Observations.

Heating of these solutions showed that from 19.11% tertiary butanol to higher percentages all systems could be converted into clear solutions. At 18.2% tertiary butanol the transition from turbid to clear could not be reached because heating was limited by the boiling point of tertiary butanol. When the tertiary butanol concentration was raised above 25.48%, the transitional temperature was not lowered significantly. At these higher concentrations, cooling from a clear solution produced, instead of opalescence, a white turbidity which altered into a coarse flocculation under continued cooling. Close examination of the opalescent system at lower tertiary butanol concentrations showed that the opalescence changed slowly into a white turbidity at lower temperatures.

Results.

After calculation of the mean values of the temperatures at which optical alterations took place, these values were plotted against the tertiary butanol concentrations of the solutions as shown in Fig. 51. The resulting diagram shows great similarity to the diagrams shown in Fig. 21 and Fig. 46 representing the optical phenomena observed

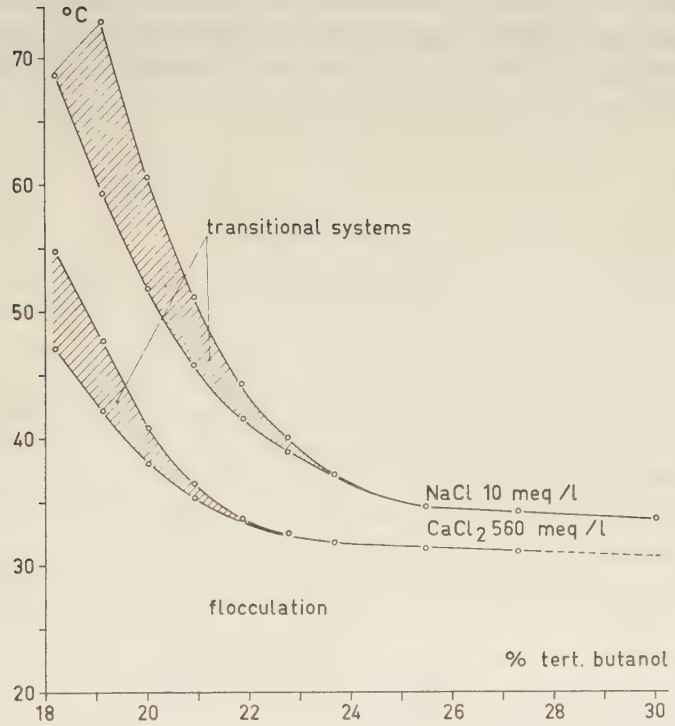


Fig. 51. Temperature-tertiary butanol diagram of spinal-cord sphingomyelin in the presence of 10 meq NaCl/L and in the presence of 560 meq $CaCl_2$/L.

with egg lecithin and spinal-cord lecithin respectively. However, the phenomena shown by sphingomyelin occur at other tertiary butanol concentrations and its behaviour at higher tertiary butanol concentrations is different: sphingomyelin crystallizes from the solution.

Discussion.

The optical phenomena of the sphingomyelin systems suggest a physico-chemical behaviour analogous to the lecithin systems. The tendency of the sphingomyelin to crystallize even in the presence of higher tertiary butanol concentrations impedes thermo-conductometric measurements.

To gain an insight into the physico-chemical properties of sphingomyelin we must in the first place try to increase its solubility. The

solubility of lecithin, as we have seen, can be raised considerably by the presence of high calcium chloride concentrations. This suggested that we might prevent crystallization of the sphingomyelin by adding calcium chloride.

b. Temperature – tertiary butanol diagram in the presence of 560 meq $CaCl_2$/L.

Solutions.

Table XXIII.

	ml	ml	ml	ml	ml	ml	ml	ml	ml
6.4% sphingomyelin in 91% tert. butanol	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54
Tert. butanol 91%	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.90	1.00
$CaCl_2$ 750 meq/L	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Aqua bi-dest.	0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.10	—
% tertiary butanol	18.20	19.11	20.02	20.93	21.84	22.75	23.66	25.48	27.30

Observations.

All these solutions could be converted into clear solutions at lower temperatures than previously found in the presence of 10 meq NaCl/L. Cooling of the clear solutions with higher tertiary butanol concentrations (23.66, 25.48, and 27.30) produced milky turbidity rather than opalescence. This phenomenon was also observed at further cooling of the opalescent system at lower tertiary butanol concentrations.

Results.

The mean values of the recorded temperatures were plotted against the tertiary butanol concentrations. To demonstrate the shift of the transitional system caused by changing the salt from 10 meq NaCl/L to 560 meq $CaCl_2$/L, we plotted both curves in the same graph (Figure 51). The influence of high calcium chloride concen-

trations is most pronounced at lower tertiary butanol concentrations, where the transitional region is broadened and shifted to lower temperatures. At higher tertiary butanol concentrations, however, the position of the more horizontal part of the curve is only slightly altered and no opalescence appears at these concentrations.

Conclusion.

High calcium chloride concentrations do not prevent the crystallization of sphingomyelin. A turbid milky region was found in the experiments with sodium chloride (10 meq/L). This turbid region was also found with calcium chloride (560 meq/L), shifted however to lower temperatures. Since the transitional region shows a considerable shift to lower temperatures, the net result of the addition of calcium chloride is only a reduction of the opalescent area.

2. Influence of gradual substitution of hen's-egg lecithin for spinal-cord sphingomyelin.

The crystallization of sphingomyelin occurring at various tertiary butanol concentrations in the presence of NaCl (10 meq/L) as well as in the presence of $CaCl_2$ (560 meq/L) restricts the physicochemical study of the properties of this phospholipid. In view of the analogous phosphoryl-choline group in sphingomyelin and lecithin, it is evident that this tendency to crystallize must be due to differences in the remaining part of the molecules.

Replacement of different amounts of sphingomyelin bij egg lecithin alters the total fatty acid composition of the system while maintaining its phosphoryl-choline composition. The influence of this replacement was first studied optically in the presence of NaCl (10 meq/L) and in the presence of $CaCl_2$ (560 meq/L).

Method.

See page 39.

Solutions.

Two series of the following solutions were prepared:

Table XXIV.

	ml	ml	ml	ml	ml	ml
6.4% sphingomyelin in 91% tert. butanol	0.54	0.43	0.32	0.22	0.11	—
6.4% lecithin in 91% tert. butanol	—	0.11	0.22	0.32	0.43	0.54
Tert. butanol 91%	0.50	0.50	0.50	0.50	0.50	0.50

To each tube of one series was added:

3.5 ml $CaCl_2$ 750 meq/L, and
0.50 ml Aqua bi-dest.

To each tube of the other series was added:

2.5 ml NaCl 20 meq/L, and
1.5 ml Aqua bi-dest.

Results.

Fig. 52 shows the graph obtained by plotting the mean values of the temperatures against the composition of the system. To give a clear picture of the composition of the system, the phospholipids are indicated by their individual concentrations in mmol/L below the abscissa.

At increasing amounts of lecithin the transitonal region is shifted towards lower temperatures and this is accompanied by a gradual disappearance of the turbidity occurring in the opalescent region of the sphingomyelin system. The presence of high $CaCl_2$ concentrations brings the whole transitional region within temperatures suitable for thermo-conductometric measurement.

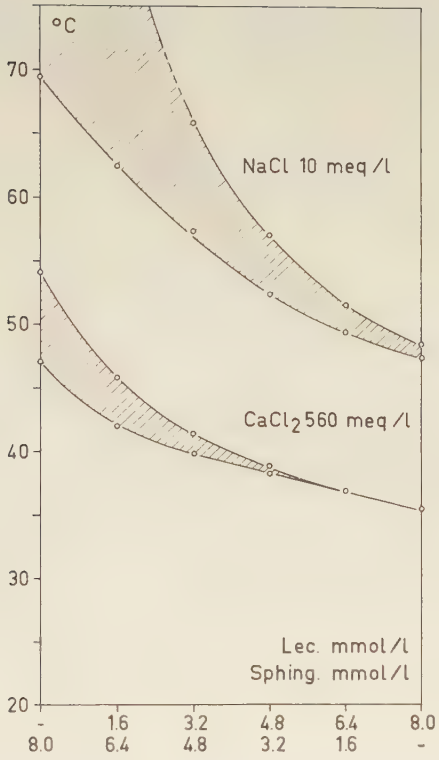


Fig. 52. Influence of gradual substitution of lecithin for sphingomyelin on the position of the transitional system (hatched area) in the presence of either 10 meq NaCl/L or 560 meq $CaCl_2$/L. Tertiary butanol concentration 18.2%.

3. Resistance factor curves of beef spinal-cord sphingomyelin and hen's-egg lecithin.

Before making serial measurements of various mixtures of sphingomyelin and lecithin, we investigated the optical phenomena observed in the pure solutions as expressed in alterations of the resistance factor. This was done in the presence of either NaCl (10 meq/L) or $CaCl_2$ (560 meq/L). To get an impression of the effect of mutual substitution, a mixture was studied containing sphingomyelin and lecithin in a ratio of 1 : 4.

Method.

See Chapter IV. For these measurements we used vessel II.

Solutions.

The following solutions were prepared by pipetting together:

Table XXV.

	I ml	II ml	III ml	blank ml	IV ml	V ml	blank ml
6.4% lecithin in 91% tert. but.	0.54	—	0.43	—	0.54	—	—
6.4% sphingom. in 91% tert. but.	—	0.54	0.11	—	—	0.54	—
Tert. butanol 91%	0.50	0.50	0.50	1.00	0.50	0.50	1.00
NaCl 20 meq/L	2.50	2.50	2.50	2.50	—	—	—
$CaCl_2$ 750 meq/L	—	—	—	—	3.50	3.50	3.50
Aqua bi-dest.	1.50	1.50	1.50	1.50	0.50	0.50	0.50

Results.

After calculating the resistance factors of the various measurements, these values were plotted against the temperatures belonging to them as shown in Fig. 53. The curves drawn through the circles represent systems containing 10 meq NaCl/L and those with the dots refer to systems containing 560 meq $CaCl_2$/L.

Examination of these curves reveals the following points:

1. Sphingomyelin, 10 meq NaCl/L, shows a considerable increase of the resistance factor. At temperatures above 29° C the resistance factors are higher than those of the corresponding lecithin system, although the resulting value at 20° C is only 45% of the value of lecithin.
2. The shift of the lecithin system to lower values under the influence of high salt concentrations corresponds to the observations described on page 64.

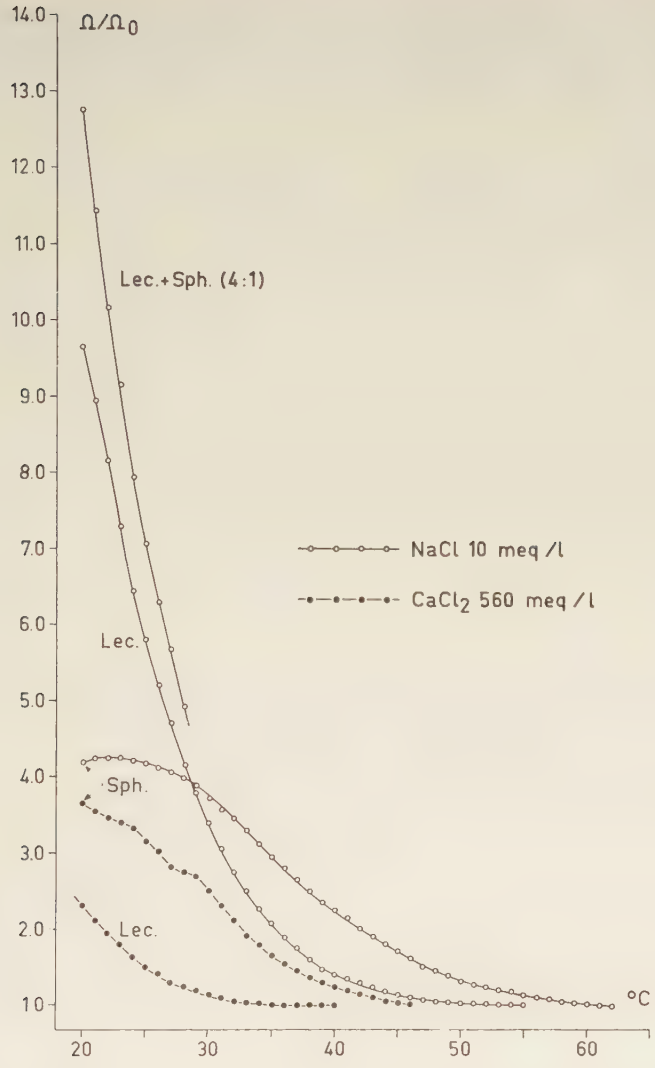


Fig. 53. Resistance factor curves of beef spinal-cord sphingomyelin and hen's egg lecithin in the presence of 18.2% tertiary butanol. Lec.-lecithin; Sph.-sphingomyelin.

3. The sphingomyelin 10 meq NaCl/L curve is shifted to a lower value in the presence of 560 meq $CaCl_2$/L; this resembles the shift of the lecithin curve although it is smaller.

4. The curve representing the mixture lecithin : sphingomyelin (4 : 1), shows much higher values than the corresponding lecithin curve.

Discussion.

Sphingomyelin, like lecithin, increases the resistance factor measured in thermo-conductometric experiments.

Expressed in resistance-factor values, sphingomyelin shows even higher values above 29° C than a corresponding lecithin system. The influence of $CaCl_2$ (560 meq/L) on the sphingomyelin system is less pronounced than on the corresponding lecithin system. Substitution of part of the lecithin by sphingomyelin results in a system with higher resistance-factor values. This phenomenon resembles those associated with the addition of cholesterol to lecithin systems. However, the resulting resistance-factor value at 20° C of the sphingomyelin/NaCl system is relatively low. In view of the optical observations it is suggested that this relatively low value is caused by gradual crystallization of the sphingomyelin as the temperature decreases.

4. **Influence of mutual substitution of sphingomyelin and lecithin on the resistance factor in the presence of 10 meq NaCl/L.**

The experiments in section 2 studied the optical phenomena of mixtures of sphingomyelin and lecithin in the presence of 10 meq NaCl/L. The regularity of the transitional region suggests a corresponding regularity in the course of the iso-resistance factor lines. To study this, solutions identical to those used for the optical experiments were measured thermo-conductometrically.

Method.

See Chapter IV.

Solutions.

The solutions were prepared according Table XXIV, page 92. Besides these solutions, a blank system was prepared containing 10 meq NaCl/L in 18.2% tertiary butanol. The solutions were chromatographed before measurement according the technique described in the Appendix. The resulting chromatogram is given in Fig. 54.

We observe on this chromatogram that the pure preparations show very small amounts of mutual contamination. Besides this contamination, the higher lecithin concentrations contain small amounts of lysolecithin.

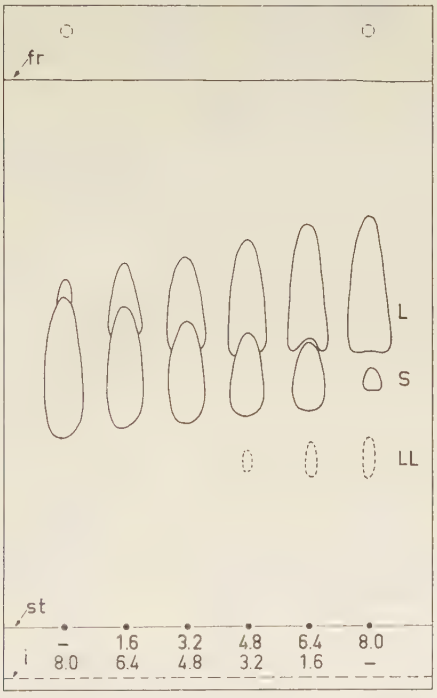


Fig. 54. Chromatogram of solutions containing sphingomyelin and lecithin in different ratios. The figures below the starting line (st) indicate either mmol/L lecithin (upper row) or mmol/L sphingomyelin (lower row). L-lecithin; S-sphingomyelin; LL-lysolecithin; fr-front of the solvent; Solvent : diisobutylketon - propionic acid – acetic acid – water, 40 : 10 : 10 : 3, by volume.

Results.

The resistance factor curves of each measured system were calculated and the resistance factors 1.5, 2.0, 4.0, 6.0, and 8.0 were selected with the temperatures belonging to them. With these data the iso-resistance factor curves were constructed as shown in Fig. 55. To complete the picture, the transition region is also indicated.

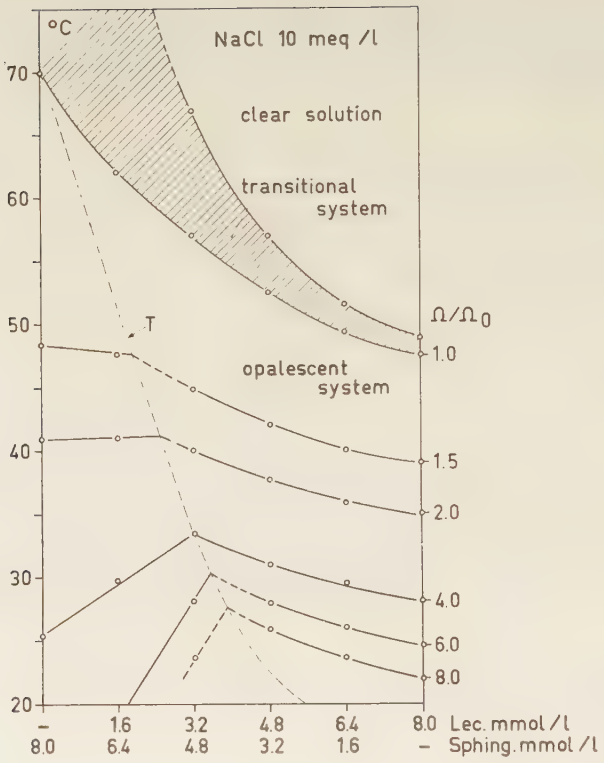


Fig. 55. Influence of mutual substitution of sphingomyelin and lecithin on the course of the iso-resistance factor lines.

As compared to the smooth course of the lower limiting line of the transitional region ($\Omega/\Omega_0 = 1.0$), the iso-resistance factor lines show an irregular course. Following a given line ($\Omega/\Omega_0 = 4.0$) from the right to the left, we find a discontinuity at a certain sphingomyelin-lecithin ratio. With cooling of a given sphingomyelin-lecithin mixture (e.g. 6 : 4) from the clear state, we find at a certain temperature a relatively smaller increase of the resistance factor. By connecting the extrapolated points of deviation, we find a line labelled T, which divides the opalescent region into two parts, the righthand one representing a zone with parallel iso-resistance factor

lines and a lefthand one in which the initial resistance factor values are reduced by crystallization of the sphingomyelin.

A more instructive picture was obtained by plotting the data of the measurement as an isothermic relation as shown in Fig. 56. By

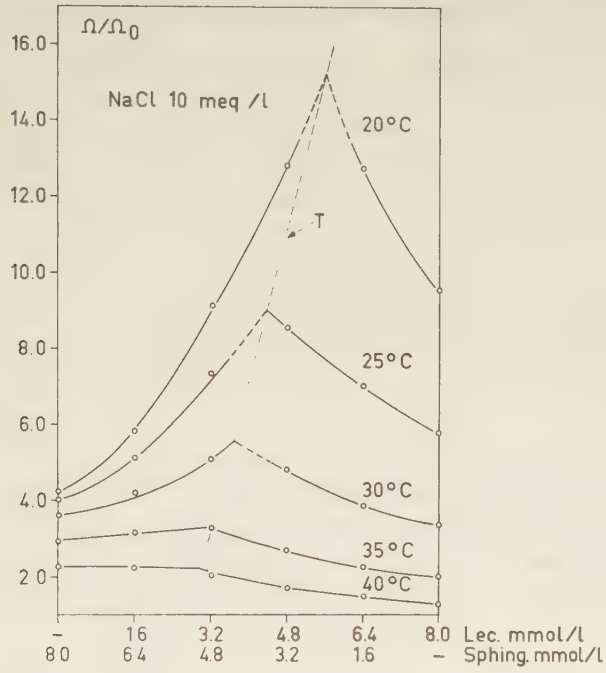


Fig. 56. Isothermic curves showing the relation between the resistance factor and the ratio sphingomyelin : lecithin. The solutions contained 10 meq NaCl/L in 18.2% tertiary butanol.

extrapolating the upward and downward slopes of the isotherms, optimal resistance factors are found at different ratios of sphingomyelin to lecithin. By connecting these extrapolated optima, the T-line reappears. To the right of this line the isotherms show a regular course and to the left the resistance factors decrease very rapidly with an increasing ratio sphingomyelin-lecithin. Higher amounts of sphingomyelin eliminate the initially higher resistance factors shown by the pure lecithin preparation. Expressed in percentages of the phospholipid concentration of the solution, we see that at 20° C an

optimum is reached at 30% sphingomyelin and at 35° C at 60% sphingomyelin.

5. **Influence of mutual substitution of sphingomyelin and lecithin on the resistance factor in the presence of calcium ions.**

The optical phenomena of mixtures of sphingomyelin and lecithin in the presence of 560 meq $CaCl_2$/L were studied in a previous experiment in which it was found that the whole transitional region was shifted to much lower temperatures (Fig. 52). This means that the thermo-conductometric measurements can be started below the boiling point of tertiary butanol from clear solutions.

Method.

See Chapter IV.

Solutions.

The solutions were prepared according Table XXIV on page 92. A blank system was prepared containing 560 meq $CaCl_2$/L in 18.2% tertiary butanol.

Results.

After calculation of the resistance factor curves, the isothermic relations were selected and plotted as shown in Fig. 57. Comparison of the isotherms of this experiment with those of Fig. 56, brings out the following points:

1. The ordinate of Fig. 56 has a more extended scale than that of Fig. 57; the resistance factors are generally lower in the presence of high $CaCl_2$ concentrations.
2. The descending slope of the isotherms at higher sphingomyelin concentrations is found to be independent of the initial state of the phospholipid system (transitional system or clear solution).
3. Considering the isotherms at 20 and 25° C in both figures from right to left, it is observed that total replacement of lecithin by sphingomyelin leads, in the presence of 10 meq NaCl/L, to

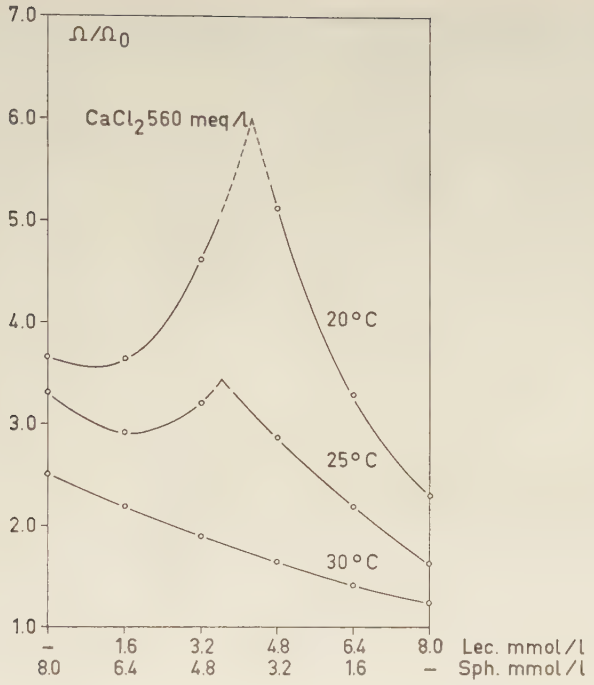


Fig. 57. Isothermic curves showing the relation between the resistance factor and the phospholipid composition in the presence of 18.2% tertiary butanol and 560 meq $CaCl_2$/L.

lower end values of the resistence factors, whereas in the presence of $CaCl_2$ 560 meq/L this total replacement causes still higher resistance factor values.

4. In the presence of $CaCl_2$ 560 meq/L the extrapolated optimal resistance factor at 20° C is related to a much higher relative percentage of sphingomyelin (50%) than the corresponding optimum in the presence of NaCl 10 meq/L (30%).
5. The influence of temperature variations on the optimal relative percentage of sphingomyelin is more pronounced in the presence of $CaCl_2$ 560 meq/L than in the presensce of NaCl 10 meq/L.

6. The fatty acid composition of hen's-egg lecithin, beef spinal-cord lecithin, and beef spinal-cord sphingomyelin.

The preceding experiments were directed to the investigation of some physico-chemical properties of hen's-egg lecithin, and lecithin and sphingomyelin from beef spinal-cord. With thermo-conductometric measurements in the presence of tertiary butanol we found a strong analogy in their properties, although differences were also found. In comparison to egg lecithin, the tertiary butanol concentration had to be raised for spinal cord lecithin. Notwithstanding higher tertiary butanol concentrations, sphingomyelin crystallizes out below a certain temperature when dissolved at higher temperatures. Since these phospholipids contain the same phosphoryl-choline group, it has already been suggested that the differences found in their properties were due to differences in the other moiety of the molecules, their fatty acid composition.

To verify this supposition the fatty acids of the preparations were analyzed by gas-chromatography *).

Results.

The fatty acid composition of the three phospholipids is shown in Fig. 58 in the form of a block diagram. The circles give the grade of saturation of the fatty acids. This diagram shows clearly that egg lecithin contains less saturated and more poly-unsaturated fatty acids than spinal-cord lecithin, while the concentration of mono-unsaturated acids are equal. Comparing these fatty acid patterns we must keep in mind that the percentages given for sphingomyelin are only related to one carbon chain. The apparent resemblance between the two phospholipids from spinal cord in relation to the grade of saturation of their fatty acids is thus not evident. Calculating the mean chain length of the fatty acids we obtain the following values (expressed in carbon atoms):

Egg lecithin 16.90,
Spinal-cord lecithin 17.09,
Spinal-cord sphingomyelin 20.02.

*) The analysis was kindly performed by Drs. C. M. van Gent, Lab. of Physical Chemistry, Leiden.

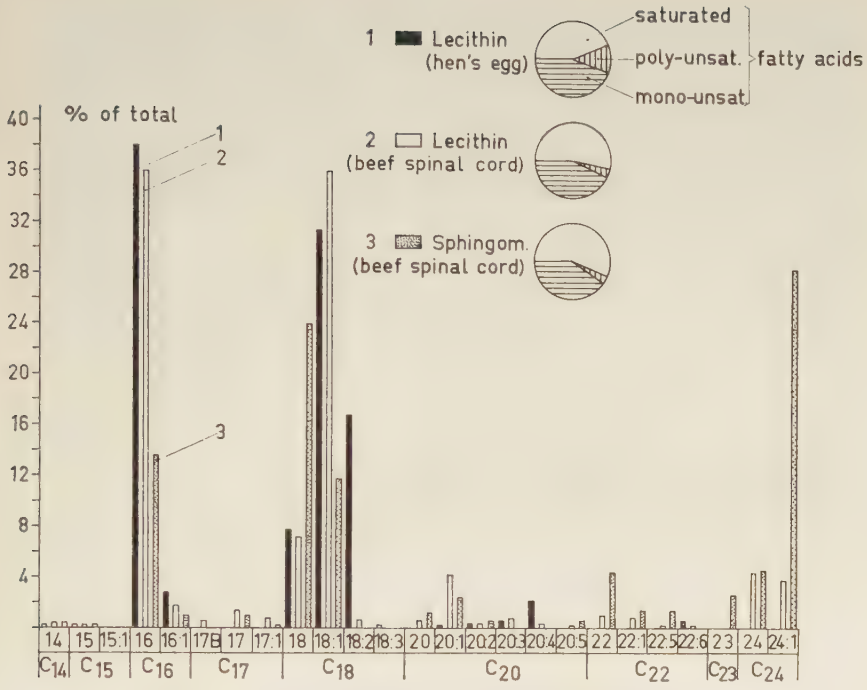


Fig. 58. Fatty acid compositions of hen's-egg lecithin, and lecithin and sphingomyelin from beef spinal-cord.

Conclusion.

It has been shown by gas chromatography that the fatty acid pattern of egg lecithin, and lecithin and sphingomyelin from beef spinal cord are very different. Our supposition that the solubility of the esterified fatty acids (estimated by chain length and saturation grade) decreases in the same sequence has proved to be valid.

IV. EXPERIMENTS WITH HEN'S-EGG CEPHALIN.

Analysis of total lipid extracts from different biological sources shows that cephalin (phosphatidyl-ethanolamine) occurs in all these materials in relatively large amounts. Cephalin was included in an investigation of the structure-forming capacity of the main phospholipids.

It proved possible to isolate sufficient amounts of cephalin from egg-yolk (see Appendix). The results of initial studies on the physico-chemical properties of these cephalin preparations were disappointing. Two factors created experimental difficulties. The first appeared to be inherent in the preparation method. The cephalin fraction is eluted from an aluminum column with a solvent mixture containing 28.5% water. This high water content of the eluent liberates sodium hydroxide from the column, causing a high alkalinity of the final cephalin preparation. Since the total cephalin fraction is concentrated by evaporation of the solvent, the resulting pH of this fraction depends on the amount of aluminum used in the column and the number of fractions taken for the preparation of a cephalin stock solution. In our preparations the pH value varied between 9 and 10, which was established for the following measurements at a lower value (pH 6) by addition of 0.01 N HCl.

The second limiting factor was the rapid deterioration of this cephalin solution. When a freshly-prepared cephalin solution in 91% tertiary butanol is stored in the dark in a refrigerator (4° C), it changes within three days into a strong yellow solution with a brown precipitate.

1. **Temperature – tertiary butanol diagram of egg cephalin.**

The experiments with lecithin revealed that the appearance of opalescence was correlated with an increase of the resistance factor. It can be expected that this correlation also exists in cephalin solutions when showing analogous optical phenomena. On the basis of this assumption (which later proved to be valid) the properties of egg cephalin solutions were studied optically during heating and cooling. For purposes of comparison, these investigations were performed at pH 6, this being the pH of corresponding lecithin solutions.

Equipment and method.

See page 39.

Solutions.

The following solutions were prepared:

Table XXVI.

	ml	ml	ml	ml	ml
6.0% cephalin in 91% tert. butanol	0.54	0.54	0.54	0.54	0.54
Tert. butanol 91%	0.85	0.90	0.95	1.00	1.05
HCl 0.01 N	1.75	1.75	1.75	1.75	1.75
Aqua bi-dest.	1.90	1.85	1.80	1.75	1.70
% tertiary butanol	24.57	25.48	26.39	27.30	28.21

Results.

In the chosen range of tertiary butanol concentrations all systems could be converted into clear solutions via a turbid transitional region by raising the temperature.

The mean values of the observed transitional temperatures are plotted in Fig. 59 against the tertiary butanol concentration.

Comparison of this diagram with that of lecithin in Fig. 21 yields the following:

1. The optical phenomena of both phospholipids during heating and subsequent cooling in the presence of tertiary butanol are qualitatively of the same character.
2. To produce these phenomena in the case of cephalin requires higher tertiary butanol percentages than are needed in the case of egg lecithin.
3. In the case of cephalin, the phenomena occur within a narrower tertiary butanol range.
4. In the cephalin diagram the transitional region is more pronounced and separates the clear solutions from the opalescent system over its entire length.
5. A dashed line is drawn in the opalescent region parallel to the lower line of the transitional region (Fig. 59). These two lines enclose a peculiar region in which the solutions are turbid when at rest and opalescent when stirred.

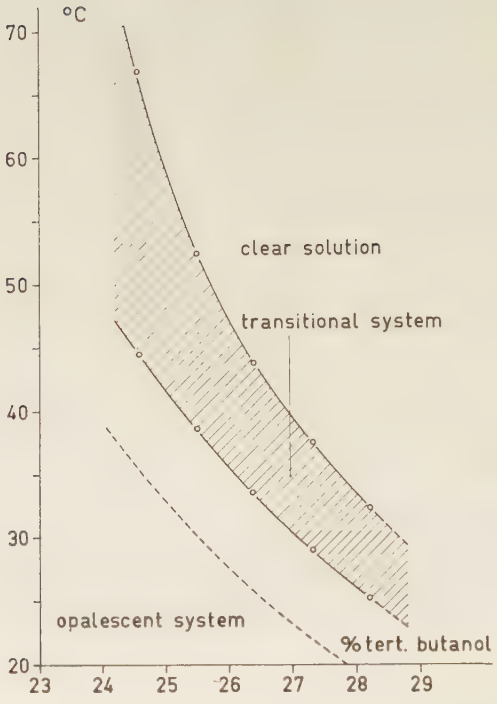


Fig. 59. Temperature-tertiary butanol diagram of hen's-egg cephalin.

2. Temperature – pH diagram of egg cephalin.

On the basis of the diagram in Fig. 59, we selected a tertiary butanol concentration of 25.48% for further investigations. At this concentration the cephalin solution shows optical changes at suitable temperatures.

Equipment and method.

See page 32.

Solutions.

The following solutions were prepared:

Table XXVII, series A

	I ml	II ml	III ml	IV ml	V ml	VI ml	VII ml
6.0% cephalin in 91% tert. but.	0.54	0.54	0.54	0.54	0.54	0.54	0.54
91% tertiary butanol	0.90	0.90	0.90	0.90	0.90	0.90	0.90
HCl 0.01 N	—	1.00	1.50	1.85	2.00	2.20	3.00
Aqua bi-dest.	3.60	2.60	2.10	1.75	1.60	1.40	0.60
pH	9.63	8.53	7.48	6.20	5.09	4.11	3.09

Inspection of these solutions showed that the first one was quite clear, the seventh turbid and the others opalescent.

Results.

The diagram in Fig. 60 is obtained by plotting the mean values of the temperatures against the pH of the solutions. The very broad

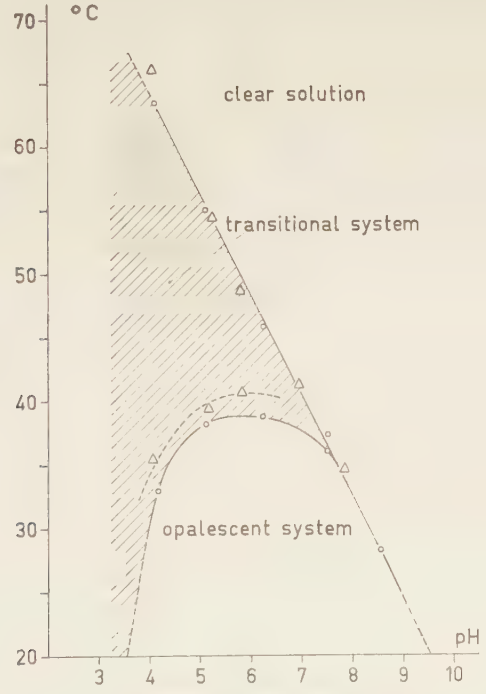


Fig. 60. Temperature – pH diagram of hen's-egg cephalin; circles indicate the relation between the transitional temperature and the initial pH; triangles indicate this relation in a subsequent experiment performed on the same series of solutions.

transitional region at lower pH values immediately attracts attention; this region persisted even at a pH of 3.09 (sol. VII). At higher pH values (above 9.5) we observe a persisting clear region. Limited by the extreme downward extension of the transitional region and the position of the clear region, the opalescent system occupies only a small area.

3. **Influence of heating and cooling on the pH of the solutions.**

At the end of the experiments described in the preceding section, the pH of the solutions was measured again. These pH values are listed in the third column of Table XXVII.

Table XXVII

solution	pH before experiment	pH after experiment
2	8.53	7.91
3	7.48	6.97
4	6.20	5.72
5	5.09	5.11
6	4.11	4.04

After these measurements the series was submitted to a second heating and cooling course and the temperatures corresponding with the optical changes were recorded. The mean values of these temperatures are shown by triangles in Fig. 60.

Discussion.

It was found that some of the pH values of a series of cephalin solutions at different pHs were lower after heating and cooling. This decrease in pH must reflect the appearance of acid substances in the solutions (e.g. fatty acids). However, paper chromatograms made prior to and at the end of the investigation failed to elucidate this problem, since neither an increase of the lysocephalin spot nor the occurrence of a fatty acid spot could be detected.

In the case of egg lecithin we also found (above pH 7) a shift of the pH after heating and cooling of the solution, but this could be related to the breakdown of lecithin into lysolecithin and fatty acids. The influence of this pH shift on the behaviour of the lecithin is almost entirely obscured by the much greater influence of the generated lysolecithin.

For cephalin, the shift in the pH values is related to a shift of the transitional temperatures, in the sense that the data of a subsequent performance fit fairly well into the initial diagram. This observation suggests that the influence of the pH on the transitional temperatures prevails over the – still hypothetical – influence of deterioration products.

4. Influence of deterioration on the temperature - pH diagram.

We have already mentioned the deterioration of the cephalin stock solution during storage in the refrigerator. Within 72 hours the solution showed a strong yellow colouration and a brown precipitate. In view of the results of the preceding experiment it may be questioned whether deterioration has any influence on the optical phenomena of the cephalin preparation. To answer this we studied these phenomena in the presence of the precipitated substance.

Method.

See Chapter IV.

Solutions.

For this investigation we used the same cephalin stock solution as used for the temperature-pH measurements in the preceding experiment. This stock solution A was used for the preparation of two other stock solutions, B and C, containing increasing amounts of precipitate obtained by storing 50 ml of solution A in the refrigerator for 72 hours. This period was sufficient to give a brown precipitate on the bottom of the storage vessel (a graduated cylinder with a ground-glass stopper). Forty ml of clear supernatant is removed by pipette and the precipitate dissolved in the remaining 10 ml by gentle

warming (stock solution C). To prepare stock solution B, corresponding in composition with solution A after 72 hours storage, 5 ml of solution C was added to 20 ml of the original supernatant of solution A. After preparation the composition of the solutions was analyzed by paper chromatography according the technique described in the Appendix. Fig. 61 gives chromatograms of these solutions together with an initial chromatogram of the fresh stock solution A.

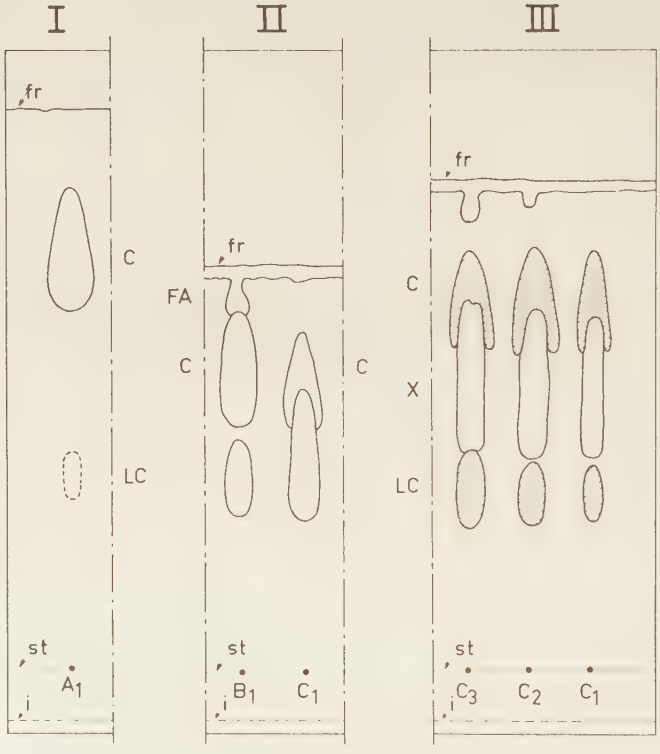


Fig. 61. Chromatogram of three egg cephalin stock solutions, A, B, and C, showing the alterations in composition during storage at 4° C. The figures appended to the letters A, B, and C indicate how many times these stock solutions were applied to the starting points. A – fresh cephalin solution; B = A after 72 hours storage at 4° C; C = B plus 5 times more deterioration products. Chromatograms I and III were run with the solvent; diisobutylketone – propionic acid – acetic acid – water, 40 : 10 : 10 : 3, by vol. Chromatogram II with the solvent: diisobutylketone – acetic acid – water, 50 : 25 : 5, by vol. C-cephalin (phosphatidyl-ethanolamine); LC-lysocephalin; FA-fatty acids; X-unknown compound.

For the study of the optical phenomena in relation to the temperature and pH of the cephalin solutions the following series were prepared:

Table XXIX, series B.

	ml	ml	ml	ml	ml	ml
Stock solution B	0.54	0.54	0.54	0.54	0.54	0.54
91% tert. butanol	0.90	0.90	0.90	0.90	0.90	0.90
HCl 0.01 N	2.10	2.00	1.90	1.80	1.70	1.50
Aqua bi-dest.	1.50	1.60	1.70	1.80	1.90	2.10
pH	4.21	4.60	5.60	6.28	6.72	7.18

Table XXX, series C.

	ml	ml	ml	ml	ml	ml	ml
Stock solution C	0.54	0.54	0.54	0.54	0.54	0.54	0.54
91% tert. butanol	0.90	0.90	0.90	0.90	0.90	0.90	0.90
HCl 0.01 N	2.20	2.10	2.00	1.90	1.80	1.60	1.40
Aqua bi-dest.	1.40	1.50	1.60	1.70	1.80	2.00	2.20
pH	4.83	5.45	6.18	6.63	6.96	7.41	7.68

Results.

Composition of the stock solutions.

Chromatograms I, II, and III, given in Fig. 61, clearly show the different composition of the three stock solutions. Chromatogram I, representing the analysis of the fresh cephalin preparation, shows besides a large cephalin spot only a small lysocephalin spot. Chromatogram II, representing the analysis of stock solution B and C, shows from B to C decreasing amounts of cephalin and increasing amounts of a substance situated lower on the chromatogram which

was taken initially for lysocephalin. However, further analysis of solution C (chromatogram III) showed that this spot could be fractionated into two spots, the lower of which appeared to be lysocephalin. (See for specific staining method page 135 in the Appendix).

The substance indicated as compound X on chromatogram III could not be determined because the following data could not be related to a known component:

1. The component occurs after storage of the cephalin preparation.
2. The precipitate formed during storage contains more compound X than the supernatant.
3. The compound shows the following staining reactions on the chromatogram:
   a. Positive with Acid Fuchsin – Uranyl.
   b. Positive with $KMnO_4$.
   c. Negative with Brilliant Green.
   d. Negative with Brilliant Green after exposure to CS_2-triethylamine.
   e. Positive with Ninhydrine.
   f. Positive with Zinzadze phosphorus reagent.
4. The place of the spot on the chromatogram.
5. The occurrence of large amounts of compound X is not accompanied by a large increase in free fatty acids.
6. The presence of larger amounts of compound X causes a relatively higher pH.

Since compound X occurs after storage of the cephalin preparation, its molecular structure must be related somehow to its parent substance. Points 3a, 3b, 3c, 3e, and 3f, also hold for cephalin, but points 3d and 4 are different.

Sub. 4.

In relation to its position on the chromatogram, compound X could have a molecular configuration between those of cephalin and lysocephalin. Only a partial breakdown of the fatty acids may be

involved, and the minor breakdown products are not detectable with our chromatographic technique. This suggestion is supported by point 5.

Sub. 3d.

The different reaction in this staining procedure indicates that the primary amine group is also involved in the alteration. Notwithstanding the presence of a positive group in the compound (point 3a), this group no longer reacts with CS_2-triethylamine.

Sub. 6.

By comparing Table XXVII (series A) with Table XXIX (series B) is is seen that the addition of a corresponding amount of HCl leads to lower pH values in series B, an indication that the initial pH of these solution was lower. Series C, however, requires more HCl than series A to attain a given pH, indicating that a higher concentration of compound X in the solution leads to a relatively higher pH.

The temperature – pH diagrams.

To obtain a clear picture of the influence of deterioration on the optical behaviour of the cephalin solutions, the diagrams of the three series were combined as shown in Fig. 62.

The most striking feature of these diagrams is the gradual disappearance of the transitional region from A to C. The position and shape of the opalescent region is much less altered, the whole region decreasing gradually with increasing amounts of deterioration products.

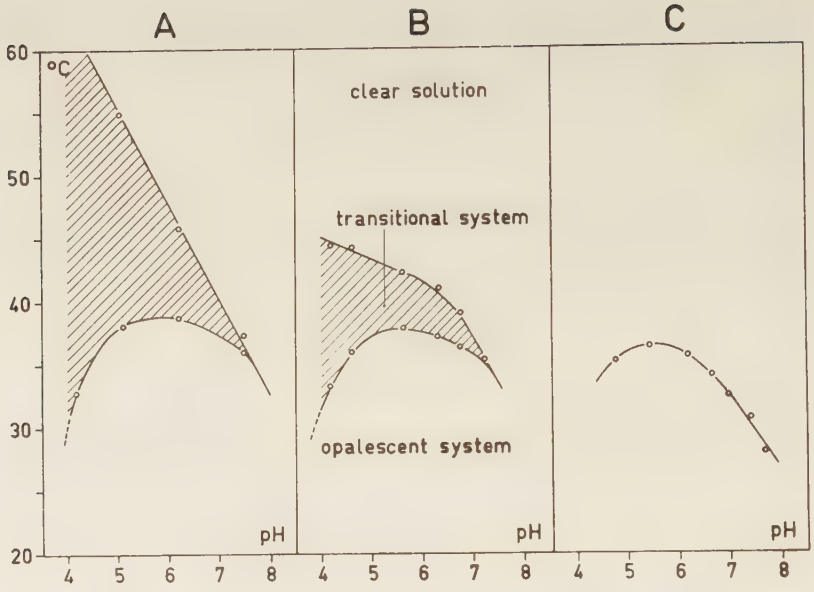


Fig. 62. Influence of deterioration on the temperature - pH diagram of cephalin. Diagrams A, B, and C represent respectively: the fresh preparation; the stored preparation (72 hours at 4° C); the preparation with a larger quantity of deterioration products.

Conclusion.

It has been found that the spontaneous deterioration of cephalin, giving rise to a high amount of an unknown compound in the solution, is reflected in the physico-chemical behaviour of the solution.

The influence of deterioration on the temperature – pH diagram is chiefly expressed in the decrease and the ultimate disappearance of the transitional region.

Since composition and physico-chemical properties of the stock solution of egg cephalin in 91% tertiary butanol altered considerably within 72 hours when stored in the refrigerator, we must conclude that this preparation is not suitable for use in thermo-conductometric measurements.

V. EXPERIMENTS WITH BEEF SPINAL-CORD CEPHALIN.

The spontaneous deterioration of hen's-egg cephalin after a short storage period creates serious obstacles to the physico-chemical study of this phospholipid. The following observations suggested that cephalin preparations from beef spinal cord are more stable.

1. During storage of a colourless cephalin stock solution at 4° (6.0% in 91% tertiary butanol) a white precipitate is formed due to crystallization of the cephalin. After 30 days no sign of brown colouration was observed.
2. A beef spinal-cord cephalin stock solution was divided into four equal portions. The first was stored in the refrigerator; the second, third, and fourth were held at room temperature. The second solution was kept in a stoppered flask, the third was bubbled with oxygen, and the fourth was bubbled with nitrogen. Paper chromatographic analysis showed that only small alterations had taken place after 72 hours, even in the oxygenated solution. After 216 hours, however, the chromatogram of the oxygenated portion showed three distinct spots: a cephalin spot reduced to half its original size, a large lysocephalin spot, and a fatty acid spot. The changes in the other portions which were kept at room temperature were similar but less pronounced. The portions stored in the refrigerator showed no alterations.

1. Temperature – pH diagram of spinal-cord cephalin.

On the basis of the results of the experiments with egg cephalin, we performed the experiments with spinal-cord cephalin in the presence of 25.48% tertiary butanol and used a concentration of 8 mmol/L.

Equipment and method.

See page 32.

Solutions.

The following solutions were prepared:

Table XXXI.

	ml	ml	ml	ml	ml	ml	ml	ml	ml
6% cephalin in 91% tert. butanol	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54
Tert. butanol 91%	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
HCl 0.01 N	0.15	0.30	0.45	0.50	0.55	0.60	0.65	0.70	0.80
Aqua bi-dest.	3.45	3.30	3.15	3.10	3.05	3.00	2.95	2.90	2.80
pH	9.65	8.91	7.24	6.54	5.86	5.21	4.37	4.04	3.61

For purposes of comparison, all solutions of this series were heated to 70° C during the temperature course, irrespective of their transitional temperatures. After cooling to room temperature, the pH of the solutions was measured again. These values differed only slightly (within 1%) from those in Table XXXI.

Results.

The mean values of the transitional temperatures were calculated and plotted against the pH of the corresponding solutions, as shown in Fig. 61. This Figure shows an arch-shaped opalescent region; at high pH values it borders on the clear region and at low pH values on the transitional region. Comparison of this diagram with the one obtained for the fresh egg cephalin shows a very great resemblance, only situated at higher temperatures.

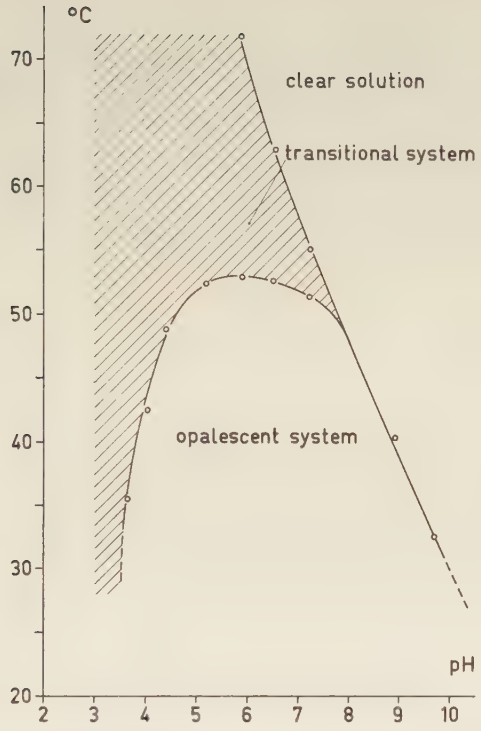


Fig. 63. Temperature - pH diagram representing optical changes during heating and subsequent cooling of a series of spinal-cord cephalin solutions at different pH values.

2. Dependence of the resistance factor of spinal-cord cephalin on the pH of the medium.

Since this cephalin preparation is relatively stable, the results of thermo-conductometric measurements will be reliable. An initial measurement of a system containing 0.6% cephalin, 25.4% tertiary butanol ,and 4 meq NaCl/L at pH 9.65, gave only a very small increase in the ultimate resistance factor (0.20), but since the arch-shaped boundary of the opalescent region in Fig. 63 suggests a strong influence of the pH of the medium on the resistance factor, measurements were done over the whole pH range.

Method.

See Chapter IV.

Solutions.

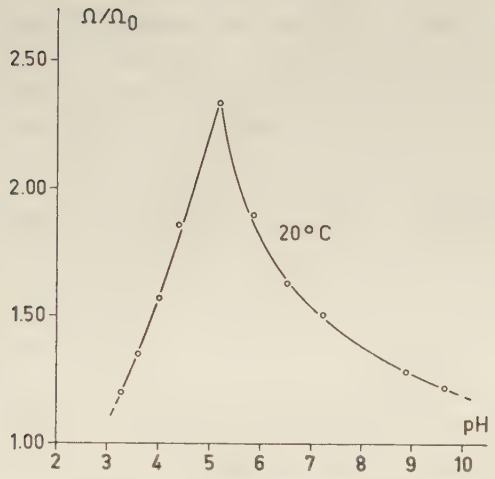
For these measurements we prepared solutions as used for the estimation of the temperature-pH relation, but with the addition of a supplementary amount of NaCl to the solutions. The blanks were prepared in the same way by replacing the cephalin stock solution by an alkaline 91% tertiary butanol solution brought to the same pH as the cephalin stock solution by addition of 0.01 N NaOH.

Table XXXII.

	I ml	II ml	III ml	IV ml	V ml	VI ml	VII ml	VIII ml	XI ml	X ml
6% cephalin in 91% tert. butanol	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54
Tert. butanol 91%	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
HCl 0.01 N	0.15	0.30	0.45	0.50	0.55	0.60	0.65	0.70	0.80	1.00
NaCl 20 meq/L	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Aqua bi-dest.	2.45	2.30	2.15	2.10	2.05	2.00	1.95	1.90	1.80	1.60
pH	9.61	8.88	7.24	6.58	5.82	5.20	4.36	4.00	3.58	3.22

Results.

After calculation of the ultimate resistance factors at 20° C, these values were plotted against the pH of the corresponding solutions as shown in Fig. 64. The curve shows a peak at pH 5.2; below this value the steep downward slope is practically linear.



Fg. 64. Isothermic curve (20° C) showing the dependence of the resistance factor on the pH of the medium of a spinal-cord cephalin system in 25.48% tertiary butanol.

Discussion.

Spinal-cord cephalin was found to show an increase of the resistance factor with a maximum at pH 5.2. This pronounced maximum indicates a great influence of the charge pattern of cephalin upon its associative state. The maximum found is in fair approximation with its I.E.P., lying between the pK_a of esterified phosphoric acid (1.5) and the pK_a of the primary amine group (9.2). However, considering this rather wide range a flatter maximum reflecting a range with amphoionic character would be expected.

3. Influence of cholesterol on the resistance factor of spinal-cord cephalin at various pH values.

From the results in the preceding section we concluded that the charge pattern of the cephalin molecules may determine the degree of association. The most favourable pH for association was found to be 5.2. The question arises of whether the presence of cholesterol can compensate a less favourable pH of the medium.

Method.

See Chapter IV.

Solutions.

From Table XXXII we selected solutions I, VI, and X. It is seen from this Table that the suitable tertiary butanol concentration is obtained by adding supplementary tertiary butanol (0.9 ml). Taking a cholesterol solution of 4.10^{-3} mmol/0.1 ml in 91% tertiary butanol, it is possible gradually to increase the cholesterol : cephalin ratio in the resulting system by replacing increasing amounts of this supplementary tertiary butanol by corresponding amounts of the cholesterol solution.

Results.

Measurements at pH 3.26.

No increase of the resistance factor value was found. Even the initial small increase of the resistance factor found in the preceding experiment (without cholesterol) was absent. Optically we found a persisting transitional region from 70 to 20° C.

Measurements at pH 5.21.

After calculation of the resistance factor curves, the isothermic relations at 20, 25, 30 and 35° C were selected and plotted as shown in Fig. 65. It was found that the maximal resistance factor increases

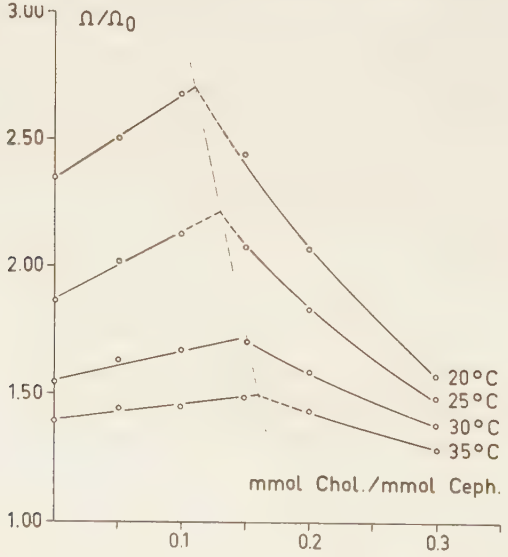


Fig. 65. Isothermic relation between the resistance factor and the ratio of cholesterol to cephalin in 25.48% tertiary butanol at pH 5.21.

only slightly when cholesterol is added. Above the ratio 1 : 10 at 20° C, the addition of cholesterol very soon causes a decrease in the resistance factor. At higher temperatures the optimal molar ratio cholesterol : cephalin is shifted slightly to a higher value, but the resistance factor values change very little.

Measurements at pH 9.6.

After calculation of the ultimate resistance factors at 20° C, they were plotted against their corresponding cholesterol : cephalin ratios as shown in Fig. 66.

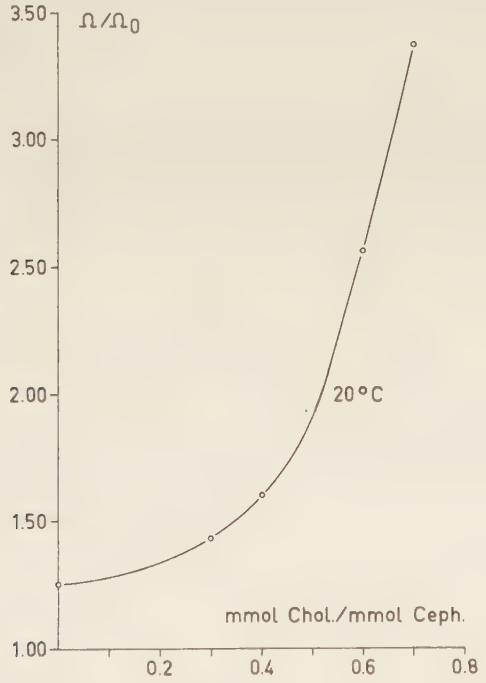


Fig. 66. Isothermic curve (20° C) showing the dependence of the resistance factor on the ratio cholesterol : cephalin in 25.48% tertiary butanol at pH 9.6.

The resulting curve shows a very rapid increase in the initially low resistance factor when the ratio cholesterol : cephalin is raised. The ratio can be increased to 7 : 10 without a sign of disturbance. Further addition of cholesterol raises the transitional temperature to unsuitably high temperatures (b.p. of tertiary butanol 82.8°) preventing the investigation of still higher ratios.

Chromatogram.

The last measured solution (Chol.-ceph., 3 : 10, pH 9.6) was chromatographed to demonstrate its composition (Fig. 67). This chromatogram shows that the cephalin preparation is contaminated with a very small quantity of phosphatidyl-serine. The added cholesterol can be recovered at the solvent front of the chromatogram after staining with trichloro-acetic acid.

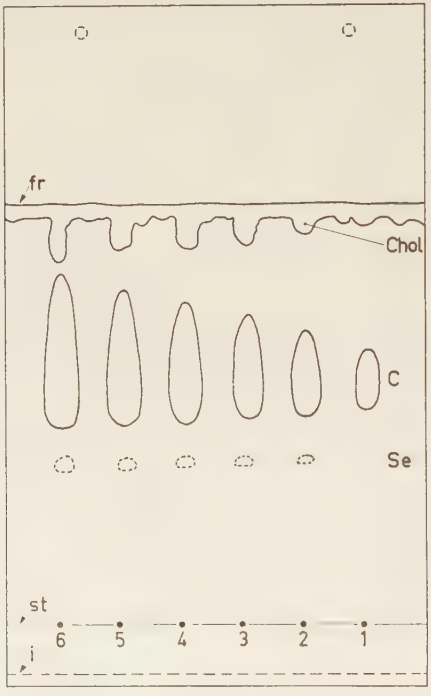


Fig. 67. Chromatogram of a beef spinal-cord cephalin system in 25.48% tertiary butanol containing cholesterol (chol. : ceph., 3 : 10) at pH 9.6. Solvent: diisobutylketon – acetic acid – water, 50 : 25 : 5, by vol. The figures below the starting points indicate how many times the system was applied to the paper. C-cephalin; Se-phosphatidyl-serine; Chol-cholesterol; f-front of the solvent; i-immersion line of the solvent; st-starting line.

Discussion.

The effect of cholesterol additions to spinal-cord cephalin systems containing 25.48% tertiary butanol proved to depend strongly on

the pH of the medium. At a low pH (3.26) the resistance factor is promptly lowered because cholesterol promotes the persistance of the transitional region. At pH 5.2 the resistance factor is initially raised by small additions of cholesterol, but this factor decreases rapidly when higher amounts of cholesterol are added. At pH 9.6 the initially low resistance factor increases rapidly with increasing amounts of cholesterol without reaching a maximum in the studied range of cholesterol : cephalin ratios (up to 7 : 10). From the experiments with egg lecithin we know that cholesterol promotes the appearance of a transitional region at higher temperatures. We must conclude from the experiments with spinal-cord cephalin that cholesterol has the same effect on this preparation, leading even to a persisting system at pH 3.26. At pH 5.2 the amount of cholesterol which can be taken up is rather small, which suggests that the cephalin is already in a condensed state. At pH 9.6 a much larger quantity of cholesterol is taken up, which suggests that the net excess of charge of the cephalin molecules is responsible for the effect, since no sign of cristallization was found.

4. **The fatty acid composition of hen's-egg cephalin and beef spinal-cord cephalin.**

The experiments with both cephalin preparations revealed a remarkable difference in their chemical stability. The very rapid deterioration of hen's-egg cephalin was accompanied by the appearance of lysocephalin and an unknown substance in the solution, as revealed by paper chromatography (Fig. 62), whereas no alterations were detected in the case of beef spinal-cord cephalin. This difference in stability could only be due to differences in fatty acid composition, which was investigated by means of gas chromatography *).

Results.

The fatty acid compositions of the fresh preparations are shown in Fig. 68 in the form of a block diagram. The grade of saturation of the fatty acids is shown in the circles.

*) The analysis was kindly performed by Dr. G. J. M. Hooghwinkel, Lab. Medical Chemistry, Leiden.

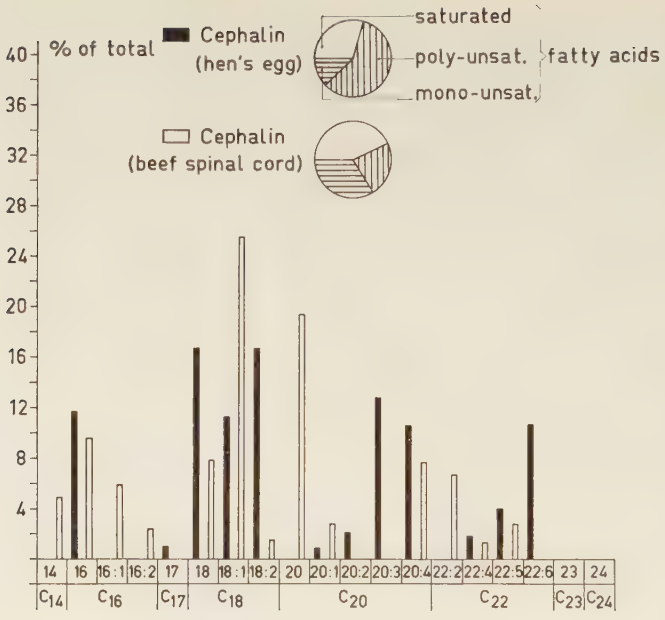


Fig. 68. Fatty acid compositions of hen's egg cephalin and of beef spinal-cord cephalin.

This diagram shows clearly that egg cephalin contains less saturated and mono-unsaturated fatty acids than spinal-cord cephalin, while the amount of poly-unsaturated acids is much greater. The mean chain length of the fatty acids is (expressed in carbon atoms):

Egg cephalin, 18.95.

Spinal-cord cephalin, 18.31.

On deterioration it was found that particularly the poly-unsaturated fatty acids disappeared, whereas the percentage of $C_{18:0}$ and $C_{18:1}$ increased. Due to its higher grade of unsaturation, egg cephalin is thus altered more rapidly.

DISCUSSION.

One of the most important unsolved problems in general cell physiology is that of the architecture of the protoplasmic membrane. The occurrence of phospholipids in this membrane is generally accepted; to explain the structural stability, however, proteins are thought to be indispensable.

Microscopical investigations (described in Chapter III) with fresh egg lecithin preparations indicated the existence of membranes in a medium consisting of water and tertiary butanol. Such systems were studied further by means of thermo-conductometric measurements (Chapter V). Purified phospholipids were isolated from fresh biological material. Lecithin, sphingomyelin, and cephalin showed qualitatively the same property of increasing the resistance of water — tertiary butanol systems when cooled gradually from a high temperature at which the systems occurred as clear, isotropic solutions. Depending on the phospholipid and the circumstances chosen, the increase in resistance differed quantitatively. The main cause of the increase in resistance is a structurally-determined hindrance of ion movements rather than a binding of conducting ions. This is demonstrated by the following experimental data.

a. Stirring a high-resistance system decreases the resistance instantaneously (page 45). Once the solution has been stirred, no recovery of the resistance factor is observed. After slight stirring the system at rest shows a somewhat lowered resistance; when stirring is continued, a small increase in the resistance occurs, followed by a further decrease. Strong stirring gives a system with the same resistance as the blank.

   The irreversible lowering of the resistance caused by stirring is a strong argument for the existence of a membrane structure in the solution. The small increase of the resistance obtained by stirring a partially disturbed system must be caused by an initial re-orientation of structural fragments.

b. Addition of cholesterol to egg lecithin systems (page 71) causes an initial increase in the resistance, which is lowered by further additions, independent of the salt concentration in the system. Cholesterol can hardly be regarded as a factor determining the binding of ions by phospholipids.

c. Replacement of lecithin by lysolecithin (page 52) or by sphingomyelin (page 91) changes the resistance even though the amount of the phosphoryl-choline groups in the solution remained the same.

The effect on the resistance caused by varying the composition of the hydrocarbon chains in the solution with maintenance of the amount and character of the charged groups cannot be understood as an influence on the binding of the conducting ions.

Factors determining membrane formation.

As factors contributing to the formation of membranes we should consider:

1. The solvent – phospholipid interaction.
2. The phospholipid – phospholipid interaction.

This differentiation, however, is rather arbitrary, since these interactions are mutually dependent.

1. *The solvent – phospholipid interaction.*

The appearance of the transitional system must be understood as a combination of two effects:

a. The solvent action of tertiary butanol.

b. The strong intermolecular force between the water molecules, which repels hydrocarbon chains.

At a certain ratio of these solvents, these two effects are ballanced in such a way that a small addition of water or lowering of the temperature converts the clear solution into a turbid system caused bij a microscopically visible phase separation. This phenomenon is not accompanied by a change in the resistance.

The influence of the tertiary butanol concentration (more strictly, the water - tertiary butanol mixing ratio) on the appearance of a transitional system when the temperature is lowered, depends on the phospholipid which is present. By comparing Figs. 21, 46, 51, and 59, we find different transitional temperatures: at 22% tertiary butanol for example:

egg lecithin	ca. 28 ° C.
spinal-cord lecithin	ca. 49 ° C.
spinal-cord sphingomyelin	ca. 60.5° C.
egg cephalin	> 90 ° C.

Since the lecithin and sphingomyelin have the same phosphorylcholine group, the nature of the long carbon chains present should be responsible for this difference. The analysis of the fatty acids shown in Fig. 58 demonstrates this correlation. The relatively high transitional temperature for egg cephalin indicates a relatively strong hydrophobic character.

2. *The phospholipid – phospholipid interaction.*

The intermolecular forces responsible for a stable membrane structure must be identical with those defining the shape of simple detergent micelles (Chapter I, Fig. 4). We should consider London van der Waals forces between the hydrocarbon chains and Coulomb forces between the charged hydrophilic groups. These factors will be strongest when:

a. The hydrocarbon chains are saturated, leading to a very dense state as revealed by the study of spread monolayers and by the study of coacervation (Chapter I, sections 4a and 4d).

b. The hydrocarbon chains are long.

c. The net charge of the structure formed is about zero, indicating that the negative and the positive groups present in the individual molecules compensate each other.

The full development of these forces will, however, prevent the formation of membranes since the resulting structure would be too rigid. Even in the case of less strong interactions, tertiary butanol is necessary to evoke the formation of membranes.

The action of tertiary butanol can be understood on the basis of its effect on common coacervated systems and on its property of evoking P-coacervation at higher temperatures when added to a suspension of smectic lecithin drops in water (Chapter I, section 4e). The action of tertiary butanol on the membrane structure has two localizations:

a. An action in the aqueous space between the micelles, resulting in an increased thickness of the water layers. This corresponds with the "opening" action of tertiary butanol on common coacervated systems.

b. An action between the hydrocarbon chains of the micelle which gives the rigid structure a sufficient flexibility to bend in three dimensions. This corresponds with the ability of tertiary butanol to cause P-coacervation.

Action b is promoted by all factors which cause a weakening of the condensing forces, enabling the tertiary butanol molecules to stay between the hydrocarbon chains. Referring to Chapter I, section 4a, these factors are:

a. Increased temperature.

b. Increased unsaturation of the carbon chains present.

c. Decreased chain length.

On this basis we can understand the crystallization phenomena observed in spinal-cord lecithin (Fig. 48), sphingomyelin (Fig. 51), and in systems containing higher amounts of cholesterol (Figs. 44 and 50).

The differences in membrane formation by different phospholipids, which result in membrane structures of different densities, suggest that the properties of natural membranes may vary with their composition. The strong effect of the pH on the cephalin membrane structure and its remarkable property of being able to compensate for a less favourable pH by taking up cholesterol, suggests that cephalin and cholesterol may have a strong influence on the properties of natural membranes. In this connection we refer to publications of De Gier and Van Deenen (1961) and De Gier (1960), who tried to correlate the lipid composition of the red-cell in various mammals to the permeability properties of the red-cells of these animals.

One difficulty in applying these results in an interpretation of the properties of natural membranes is that the systems studied contained tertiary butanol. Studies in more physiological media are necessary. The present comparative study has demonstrated that differences in the molecular structure of various natural phospholipids lead to a different membrane structure *in vitro*.

At the end of this discussion we cannot omit mentioning the recent investigations of Mueller et al. (1962a, b: 1963) and of Thompson (1964). Mueller studied thin complex underwater films composed of phospholipids and simple lipids. Thompson investigated the formation of a thin film between two aqueous phases developed from a mixture of lecithin and tetradecane, dissolved in methanol-chloroform. These experiments, like ours, indicate that a relatively stable lipid membrane structure can exist between two aqueous phases.

SUMMARY.

The aim of our investigation was to determine whether the main natural phospholipids (lecithin, sphingomyelin, and cephalin) could exist as a membrane structure between two aqueous media without the aid of other covering substances. If this could be shown, the role of the phospholipids as structural elements and their influence on permeability would be clarified.

To obtain adequate quantities of freshly-prepared phospholipids for this work, we developed a rapid method for the extraction, separation, and purification of these substances from natural sources.

Microscopical and thermo-conductometrical investigations revealed the existence of membrane structures in water – tertiary butanol mixtures. By studying egg lecithin, spinal-cord lecithin, spinal-cord sphingomyelin, and spinal-cord cephalin thermo-conductometrically, it has been demonstrated that these substances can form stable membrane structures in a water – tertiary butanol environment. (The same phenomenon was found with rat-liver lecithin and soy-bean lecithin; these experiments have not been described in this thesis.) The resulting membrane structures showed different resistance factors depending on their fatty acid composition.

Investigations with egg lecithin showed that the density of the membrane structure is dependent on the temperature of the system. The density of the membranes was limited by the lecithin concentration. Up to 12.8 mmol/L, the resistance increased, to remain the same with a further increase of the lecithin concentration. This phenomenon could not be related to alterations in the ratios of lecithin to NaCl or lecithin to tertiary butanol.

The action of snake venom lecithinase on a membrane structure could be accounted for by the appearance of lysolecithin and the disappearance of lecithin. Quantitative studies revealed that at a molar ratio of lysolecithin to lecithin of 3 : 10, membrane formation was totally suppressed.

Low salt concentrations were found to promote membrane format-

ion, while high concentrations suppress it. Further investigations in a medium containing physiological salt concentration are required.

The pH of the medium containing egg lecithin has only a slight influence on membrane formation, at least in the range between pH 5 and about pH 9. The upper limit of this pH-range could not be determined with precision. At pH values above 7, the composition of the system changes due to the appearance of lysolecithin and fatty acids.

Addition of cholesterol causes a much denser membrane structure up to a molar ratio of 1 cholesterol : 4 lecithin, in the presence of 280 mmol $CaCl_2$/L.

Studying spinal-cord lecithin, it was found that below 22.75% tertiary butanol, crystallization occurs. This was related to the greater percentage of saturated fatty acids in spinal-cord lecithin. In the presence of high calcium chloride concentrations, the optimal molar ratio of cholesterol : spinal-cord lecithin was found to be 1 : 3.6.

The investigation of sphingomyelin was limited by the fact that sphingomyelin crystallized from the solution at lower temperatures; this phenomenon was independent of the tertiary butanol concentration. This indicates that the action of tertiary butanol is completely abolished below a certain temperature due to very strong condensing factors (high grade of saturation and long carbon chains). Investigations with different ratios of sphingomyelin and egg lecithin showed the great influence of temperature on the optimal mixing ratio; this ratio appeared to depend on the salt concentration as well.

The experiments with cephalin preparations from hen's eggs were disappointing and unreliable because of the very rapid deterioration of such preparations. Lysocephalin and a large quantity of a still-unknown substance appear in the solution after standing. This rapid deterioration was related to the large unsaturated fatty acid content of egg cephalin. The position of the transitional region of cephalin derived either from hen's eggs or from beef spinal cord was found to depend greatly on the pH of the medium; at pH values below 4 this region extends from 65° to about 20° C. Membrane formation by spinal-cord cephalin depends strongly on the pH of the medium, giving the most condensed structure at pH 5.2. This would mean that natural membranes containing cephalin carry an

excess negative charge, causing a lower density of the membrane. Addition of cholesterol to cephalin systems at different pH values revealed that a less favourable pH of 9.6 could be compensated for by cholesterol, leading to an even much denser structure than shown by the optimal cholesterol-cephalin system at pH 5.2.

From these experiments it is concluded that phospholipids are substances which form membranes spontaneously. Differences in resistance may be correlated either with differences in fatty acid composition or with differences in the hydrophilic group of the phospholipids. Addition of cholesterol leads to a much denser structure, especially at certain ratios of cholesterol to phospholipid. This effect appeared to depend on the charge pattern of the hydrophilic group of the phospholipid present. Small amounts of salts promote an increase in the density of the membranes as contrasted with higher amounts. Lysolecithin shows a strong suppressing action.

SAMENVATTING

Een der belangrijkste problemen in de algemene celfysiologie is de moleculaire structuur van de protoplasma membraan. Experimentele gegevens (zie Hoofdstuk I) hebben het waarschijnlijk gemaakt, dat de relatief grote hoeveelheid fosfolipiden in biologisch materiaal nauw gecorreleerd is met deze membraan structuur. Met ons onderzoek hebben we nagegaan of fosfolipiden (lecithine, sfingomyeline en kefaline), geïsoleerd uit biologisch materiaal, zelfstandig membraan structuren kunnen vormen.

Om over voldoende fosfolipiden te beschikken, ontwikkelden we allereerst een snelle bereidingsmethode. Na een uitgebreid vergelijkend onderzoek vonden we dat een mengsel van diethylether - tertiair butanol (1 : 1)de beste resultaten gaf bij een geconcentreerde extractie van vers materiaal. (1 gew. materiaal met 3 vol. extractie vloeistof). De extractie wordt gevolgd door een kolomzuivering waarbij het totale geconcentreerde extract gechromatografeerd wordt over Al_2O_3. Dit onderzoek is uitvoerig behandeld in de Appendix.

In Hoofdstuk III hebben we de microscopisch waarneembare veranderingen beschreven, die optreden in een lecithine oplossing (in 45.5% tertiair butanol) bij langzaam verdunnen met water. De microfoto in Figuur 12 toont als eindstadium van deze verdunning membraanstructuren (stadium 9), ontstaan via verschillende overgangsstadia.

Na de constructie van een geschikte apparatuur (Hoofdstuk IV) werden deze systemen (fosfolipide - water - tertiair butanol) nader onderzocht met de thermo-conductometrische methode (Hoofdstuk V). Bij een juiste mengverhouding water - tertiair butanol verschijnen fosfolipiden bevattende systemen bij hogere temperaturen als heldere isotrope oplossingen. Bij langzaam afkoelen verandert de heldere oplossing in een opalescent systeem, al of niet via een troebel overgangsstadium (transitional system, Fig. 8). De opalescentie gaat gepaard met een toename van de weerstand. Als oorzaak van dit verschijnsel komen de volgende mogelijkheden in aanmerking:

1. De ionen worden gebonden aan de afzonderlijke fosfolipiden bij verlaging van de temperatuur.
2. De ionen worden gehinderd door een slecht geleidende membraanstructuur die in de oplossing ontstaat bij verlaging van de temperatuur.

Uit de volgende experimentele gegevens concludeerden we, dat de tweede oorzaak de meest waarschijnlijke is.

a. Wanneer een systeem met een hoge weerstand geroerd wordt neemt de weerstand plotseling af. Na het roeren zien we geen herstel van de oorspronkelijk hoge weerstand. Na kort en langzaam roeren is de weerstand gedeeltelijk verlaagd; indien het roeren wordt hervat bemerken we primair een geringe toename van de weerstand, gevolgd door een verdere sterkere afname. Bij sterk roeren wordt de weerstand gelijk aan de weerstand van het systeem zonder fosfolipide.

b. Toevoeging van cholesterol aan ei-lecithine bevattende systemen geeft aanvankelijk een weerstandsverhoging die bij verdere toevoeging van cholesterol weer afneemt. Dit verschijnsel is onafhankelijk van de zoutconcentratie in het systeem.

c. Vervanging van lecithine door lysolecithine of door sfingomyeline verandert de weerstand van het systeem ofschoon het aantal fosforyl-choline groepen in de oplossing gelijk blijft.

Sub. a. De irreversibele verlaging van de weerstand na roeren is een sterk argument voor het bestaan van membraanstructuren in de oplossing. De geringe weerstandsverhoging die men primair vindt bij het opnieuw roeren van een gedeeltelijk afgebroken systeem wordt verklaard als een aanvankelijke reorientatie van structuur fragmenten.

Sub. b. Cholesterol kan moeilijk beschouwd worden als een stof die de binding van ionen beïnvloedt.

Sub. c. De verandering van de weerstand ten gevolge van een veranderde samenstelling van de aanwezige koolstofketens kan niet worden verklaard door een invloed op de binding van ionen.

Bij het onderzoek van ei-lecithine vonden we, dat de weerstand van het systeem afhankelijk was van de temperatuur en van de lecithine concentratie (Fig. 33). Tot 12.8 mmol lecithine per liter

nam de weerstand toe, boven deze concentratie vonden we geen verdere toename. Dit verschijnsel konden we niet verklaren door veranderingen in de verhoudingen van lecithine : natrium chloride of van lecithine : tertiair butanol.

De lytische werking van slangengif-lecithinase op de membraanstructuren werd aangetoond en verklaard uit het ontstaan van lysolecithine en het verdwijnen van lecithine (Fig. 25 en Fig. 26). Uit kwantitatieve onderzoekingen bleek dat bij een moleculaire verhouding van lysolecithine en lecithine als 3 : 10, geen membraanvorming optrad (Fig. 28).

Lage zoutconcentraties bevorderen de vorming van membranen, terwijl hoge concentraties de vorming tegengaan (Fig. 36, 37 en 38).

De invloed van de pH op de membraanvorming werd bestudeerd, hieruit bleek dat in een pH gebied van 5 tot ongeveer 9 de resulterende weerstandsverhoging onafhankelijk was van de pH (Fig. 41). De bovengrens van dit gebied kon niet nauwkeurig bepaald worden aangezien boven pH 7 de samenstelling van het systeem veranderde door afbraak van lecithine in lysolecithine en vetzuren (Fig. 42).

Toevoeging van cholesterol veroorzaakte een veel compactere membraanstructuur. Een optimale weerstandsverhoging vonden we bij een molecuulverhouding van cholesterol : lecithine als 1 : 4 (Fig. 44).

Bij het onderzoek van ruggemerg-lecithine bleek dat dit fosfolipide uitkristalliseerde bij lagere tertiair butanol concentraties dan 22,75% (Fig. 48). Dit verschijnsel wordt veroorzaakt door de aanwezigheid van een hoger percentage verzadigde vetzuren in ruggemerg-lecithine in vergelijking met ei-lecithine. De optimale mengverhouding van cholesterol en ruggemerg-lecithine kon worden onderzocht in een milieu met een hoge $CaCl_2$ concentratie. Deze verhouding bleek ongeveer gelijk aan die voor ei-lecithine, namelijk 1 : 3,6 (Fig. 50).

Het onderzoek van sfingomyeline werd bemoeilijkt door de sterke kristallisatie-neiging van dit preparaat. Dit verschijnsel bleek onafhankelijk te zijn van de tertiair butanol concentratie (Fig. 51). Uit onderzoekingen bij verschillende mengverhoudingen van sfingomyeline en ei-lecithine vonden we een sterke in-

vloed van de temperatuur op de optimale mengverhouding (Fig. 56), bovendien bleek deze verhouding afhankelijk te zijn van de zout concentratie in het milieu (Fig. 52).

De onderzoekingen met verscheidene kefaline preparaten bereid uit kippeneieren waren zeer teleurstellend. Bij bewaren van deze preparaten trad een spontaan bederf op waarbij lysokefaline ontstond en een grote hoeveelheid van een nog onbekende stof (Fig. 61). Dit snelle bederf wordt veroorzaakt door het sterk onverzadigde karakter van de vetzuren in ei-kefaline. De ligging van het troebele overgangsgebied bleek zowel voor ruggemerg-kefaline als voor ei-kefaline afhankelijk te zijn van de pH van het medium (Fig. 60 en Fig. 63); bij pH waarden lager dan 4 strekt dit gebied zich zelfs uit van 65° tot ongeveer 20° C. De vorming van membraanstructuren door ruggemerg-kefaline wordt sterk beïnvloed door de pH van het milieu; bij pH 5.2 werd de dichtste structuur gevonden (Fig. 64). Dit betekent, dat biologische membranen die kefaline bevatten een negatieve overschotslading dragen. Onderzoek van kefaline - cholesterol systemen bij verschillende pH's toonden aan dat een ongunstige pH (9.6) gecompenseerd kon worden door cholesterol; dit leidde zelfs tot veel compactere structuren dan bij de optimale pH van 5.2 (Fig. 66).

Uit ons onderzoek moeten we concluderen dat fosfolipiden spontaan membranen kunnen vormen in een water - tertiair butanol milieu. Kwantitatieve verschillen in de weerstand der verschillende fosfolipide systemen kunnen herleid worden tot verschillen in de vetzuur samenstelling af tot verschillen in de hydrofiele groep der moleculen. Dit is een aanwijzing, dat de eigenschappen van biologische membranen zullen verschillen naarmate de fosfolipide samenstelling verschillend is. Het sterke pH effect op de kefaline membraanstructuur en de opmerkelijke eigenschap om een minder gunstige pH te kunnen compenseren door opname van cholesterol, duidt op een belangrijke invloed van kefaline en cholesterol op de eigenschappen van natuurlijke membranen. In dit verband wijzen we op de publicaties van De Gier en Van Deenen (1961) en van De Gier (1961), die trachtten een verband te leggen tussen de lipide samenstelling van de erythrocyten van bepaalde zoogdieren en de permeabiliteit van hun erythrocyten.

De directe interpretatie van onze resultaten als eigenschappen

van natuurlijke membranen wordt bemoeilijkt door het onfysiologische milieu (water - tertiair butanol) waarin onze metingen hebben plaats gevonden. Voor de toekomst stellen we ons dan ook voor dit onderzoek voort te zetten met media van een meer fysiologische samenstelling. Wanneer we dit onderzoek voorlopig beschouwen als een vergelijkende studie van verschillende biologisch voorkomende fosfolipiden dan hebben we hiermede aangetoond dat een verschil in moleculaire structuur aanleiding geeft tot het ontstaan van verschillende membraanstructuren *in vitro*.

APPENDIX.

A. PAPER CHROMATOGRAPHY.

In 1956 Marinetti and Stotz introduced their paper chromatographic method for the separation of small amounts of phopholipid mixtures into their constituents. The paper was previously impregnated with silica and the solvent was diisobutylketon – acetic acid – water (50 : 25 : 5, v/v). This method provides very good separation, but the impregnation of the paper is rather complicated and the results are not always reproducible. This led us to develop a simplified impregnation method (Bungenberg de Jong and Hoogeveen; 1960c, d, e; 1961a, b).

1. **Impregnation of the paper.**

The chromatographic paper (e.g. Schleicher and Schüll, 2043b) is impregnated by pulling it through a solution of $SiCl_4$ in CCl_4 (e.g. 2% solution). The water in the paper reacts with the $SiCl_4$ to form a thin layer of SiO_2 around the paper fibers. After hanging in the air for 70 hours, for evaporation of the HCl formed, the paper is ready for use. This easy preparation method produces a paper with constant properties.

2. **Chromatographic method.**

With a 5 μl micropipette samples are applied to the starting points on $SiCl_4$-impregnated paper. After the spots have dried, the paper is fixed in the framework of the slit-feeding apparatus (Bungenberg de Jong and Hoogeveen; 1960a, b) and placed in the glass container belonging to it. Chromatography is performed in a dark room at a constant temperature of 20° C. The solvent mixture is a modification of the one given by Marinetti and Stotz (1956): we introduced propionic acid and decreased the amounts of the other constituents, thus: diisobutylketon – acetic acid – propionic acid – water (40 : 10 :

10 : 3, v/v). This solvent gave better separation of the spots in lower locations. Chromatography is performed to a given height, then the paper is taken out of the apparatus and after drying, stained with Acid Fuchsin (see below).

The spot area increases significantly with increasing concentration. This relationship was studied by Bungenberg de Jong and Hoogeveen (1960d) who determinded that the log concentration was proportional to the log of the area (Fig. 69).This chromatographic

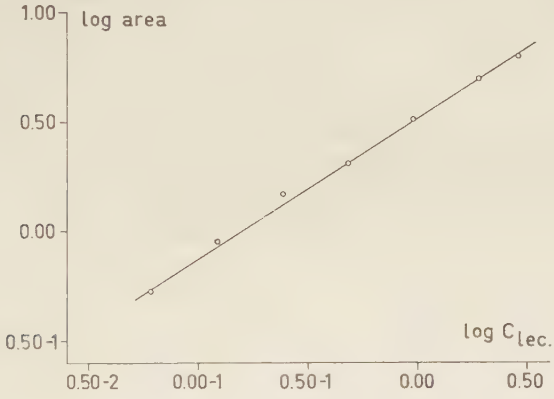


Fig. 69. Relation between the lecithin concentration of a solution and the area of the resulting lecithin spot on $SiCl_4$-impregnated chromatographic paper (Schleicher & Schüll, 2043b).

method enables qualitative analysis of a lipid mixture, but also permits an approximately quantitative analysis done by measuring the area of the spots with a planimeter.

3. Detection of the various components on paper chromatograms.

In 1959, Hooghwinkel, Hoogeveen, Lexmond and Bungenberg de Jong published a summary of the spot tests used for the detection of phospholipids on paper chromatograms. In the same year, Bungenberg de Jong and Van Someren (1959) published their work on the use of Acid Fuchsin and uranyl nitrate in staining chromatograms of phospholipids (Tricomplex-staining method). This qualitiative test

was given a quantitative basis by Hooghwinkel and Van Niekerk (1960) and Van Niekerk (1962). With this technique the phospholipids are stained with a negative stain (e.g. Acid Fuchsin 0.002%), while the negative charge of the phosphate group is blocked by a cation with a high affinity for this group (e.g. UO_2^{++}, uranyl nitrate 0.2%) at pH 2 (HCl 0.01 N.). After staining for 45 min. the chromatogram is washed with 0.01 N HCl containing 0.2% uranyl nitrate (5 min) and after blotting with filter paper, dried with the warm air from an electric hair dryer.

Proteins are stained too, but remain at the starting point. The simple lipids are not stained in this way; these components can be detected at the front line of the chromatogram by exposing it to iodine vapour in a closed reservoir. The fatty acids, also situated at the front of the chromatogram, and other negative components can also be detected by staining them with Brilliant Green (0.01%). The phospholipids and the more acid components can be stained simultaneously in a mixed bath (Bungenberg de Jong, 1961) containing Acid Fuchsin 0.02% Brilliant Green 0.01% and uranyl nitrate 0.04% in acetic acid 0.1 N.

A very simple method for differentiating between phospholipids containing choline and ethanolamine is described by Bungenberg de Jong (1961) by exposing the chromatogram to the vapours of CS_2 and triethylamine, after which the chromatogram is stained with the mixed staining bath. Cephalin and phosphatidyl-serine appear as green spots and the other phospholipids are stained red.

The detection of cholesterol can be performed by pulling the chromatogram through a mixture of trichloro-acetic acid and water 1 : 1 (w/v), followed by heating at 90° C. After ten minutes the cholesterol spot shows a blue colour which changes within 30 minutes via green into a red colour.

B. THE EXTRACTION, ISOLATION, AND PURIFICATION OF PHOSPHOLIPIDS.

To prepare natural complex lipids from different sources, earlier techniques made use of salt (complex) formation (Levene and Rolf, 1927; Pangborn, 1941) or of the different solubilities in various organic solvents (Folch, 1942; Scholfield and Dutton, 1957). More

recently, chromatographic separation and purification techniques have been developed (Rhodes and Lea, 1957; Hanahan, 1961). However, the procedures described require an inconveniently amount of time, mainly because (1) a great excess of extraction liquid has to be used, and (2) the chromatographic adsorbent has a relatively low adsorption capacity.

Because oxidation and enzymatic breakdown of phospholipids are to be expected (Hanahan and Charkaff, 1947a, b; Bungenberg de Jong and Hoogeveen, 1961c), we developed a rapid preparation technique with a high yield.

The extraction procedure is based on a material-solvent ratio of 1 to 3 (wet weight : ml) while the subsequent column chromatographic isolation and purification method could be performed with a loading of 1 : 20 (1 gram phospholipid : 20 grams Al_2O_3). This provides us with a very useful technique for the production of larger amounts of lecithin, sphingomyelin, and cephalin.

I. THE EXTRACTION METHOD.

In designing an extraction procedure for fresh tissue based on a high ratio of material to solvent, various complications may be encountered, such as:

a. The high water content of biological materials. Since many of the applied organic "fat" solvents are immiscible with water, their extraction capacity is reduced in the presence of water. The combination: water, lipid, and organic solvent creates favourable circumstances for the formation of stable emulsions.

b. The large amount of proteins in biological material also disturbs the extraction procedure, since proteins cause emulsification.

c. The complex lipids are partly bound in the tissue and these bound lipids are less extractable with a pure solvent.

d. Some organic liquids are also solvents for non-lipid components.

e. Enzymatic breakdown of the lipids may occur during the preparation.

f. The restricted solubility of lipids in some organic solvents.

g. Deterioration of the lipids may occur during the preparation.

In view of these complicating factors, some simplifications were required: (1) hen's egg-yolk was chosen as material because it contains large amounts of phospholipids; (2) the proteins were coagulated in advance, thus excluding the disturbing factors mentioned under b and e; (3) the number of extraction liquids was limited. Guided by their boiling points, we selected some organic solvents and arranged them as shown in Table XXXIII.

Table XXXIII.

Some organic solvents with low boiling points.

	Group A		Group B	
no.	immiscible with H_2O	B.P. °C	miscible with H_2O	B.P. ° C
1.	diethyl ether	34.6	acetone	56.5
2.	petroleum ether	40-60	methanol	64.7
3.	chloroform	61.2	ethanol	78.4
4.	carbon tetra-chloride	76.8	iso-propanol	82.3
5.	ethyl acetate	77.2	tertiary butanol	82.9
6.	benzene	79.6	propanol	97.8
7.	iso-butanol	108.4		

The solvents of Group A are, in general, better lipid-solvents than those of Group B, but they are less effective when water is present (factor a). In order to tolerate a higher amount of water, Group B solvents must be added to Group A solvents. Another reason for adding solvents of Group B is their ability to disrupt the linkage between lipids and proteins (factor c).

a. DEVELOPMENT OF AN EXTRACTION PROCEDURE FOR PRE-COAGULATED MATERIAL.

In order to avoid loss, deterioration, and undue expenditure of time, the extraction solvent had to combine a high extraction capacity with a low boiling point and to tolerate a large amount of water.

Material.

Yolk powder was prepared as follows:
Hen's eggs were boiled for 30 minutes, after which the yolks were removed and cut into small pieces. After freezing for 3 hours at —13° C, these pieces were ground to a fine powder at the same temperature in a Waring blender.

1. The extraction capacity of water-miscible solvents in relation to the water content of the extraction system.

From preliminary test-tube experiments it was known that water gave emulsions when shaken with fresh yolk powder and that the solvents of Group B could suppress this emulsification. For a material – solvent ratio of 1 : 3, w/v, the ultimate extraction mixture must be compatible with 20-25% water.

We first studied the solvents of Group B for their extraction capacity and their capacity to suppress emulsification in relation to the amount of water present in the extraction system (substrate plus solvent), employing an extraction technique followed by paper chromatography. The extraction was evaluated by measuring the areas of the lecithin spots on the chromatogram with a planimeter.

Solvents.

Acetone, methanol, ethanol, and tertiary butanol.

Method.

Two hundred mg of powdered egg yolk was weighed into a series of seven centrifuge tubes with ground-glass stoppers and a series of seven extraction liquids was prepared consisting of an organic solvent with increasing percentages of water. The total amount of each liquid was 12 ml, the water content being increased in 2 ml steps form 0 ml tot 12 ml. From each of these solutions 3.5 ml was pipetted into the corresponding centrifuge tube. The tubes were provided with small glass iron-core stirring rods and placed on a magnetic stirring device for 60 min. The tubes were then centrifuged for 10 min., after which the supernatant was chromatographed and the chromatogram stained. (Appendix page 140).

Results.

The water content of yolk powder being 50%, the percentages of water in the extraction liquids containing acetone, methanol, or tertiary butanol are: 2.8, 17.4, 33.9, 50.4, 66.9, 83.5, 100. For ethanol

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they are: 6.8, 20.7, 36.6, 52.4 68.2, 84.2, 100. Examination of the supernatant of the various series after centrifugation generally shows that with increasing percentages of water the initially very yellow clear solutions become less coloured and change at a certain water percentage into a white turbid suspension with yellow fatty drops on the surface.

A chromatogram of the actone-water extraction is given in Fig. 70. The chromatogram shows spots for lecithin, cephalin, sphingomyelin, and lysolecithin. At the front, spots of simple lipids can be detected, whereas protein spots make their appearance to the right at the starting line, indicating that despite the precoagulation, proteins are dispersed at higher water percentages. In Fig. 71 we

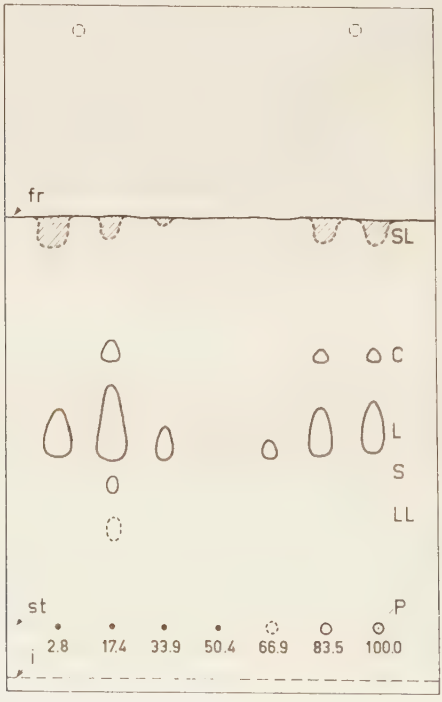

Fig. 70. Chromatogram showing the results of egg yolk powder extraction with acetone-water mixtures containing increasing percentages of water, as indicated at the starting points. L-lecithin; C-cephalin; S-sphingomyelin; LL-lysolecithin; SL-simple lipids; P-protein.

have plotted the spot area of lecithin against the water percentages in the extraction media of the different series. In these graphs the presence of turbid supernatants at higher water percentages is indicated by hatching.

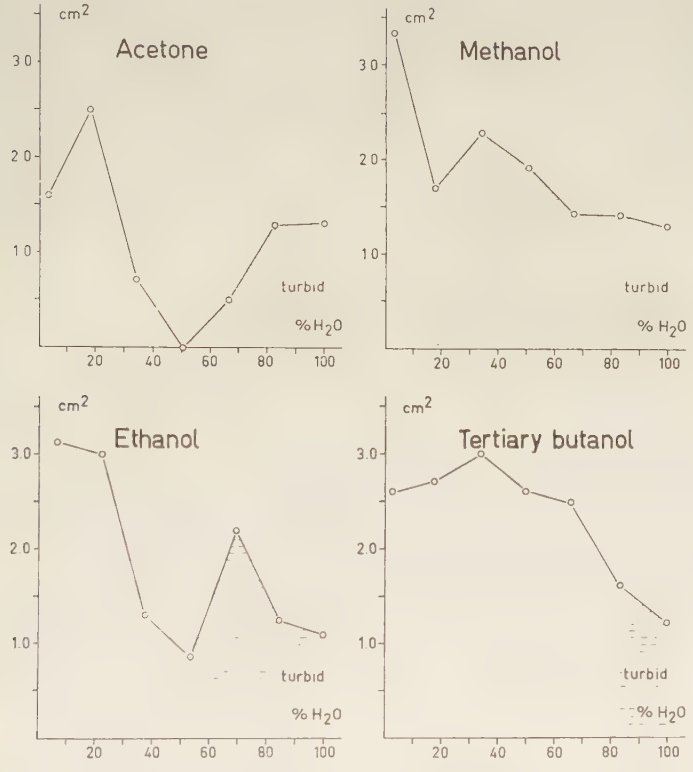

Fig. 71. The spot area of lecithin (in square cm) in relation to the percentage of water in the extraction system. The areas are measured on chromatograms made from the supernatants after extraction and centrifugation. Turbidity of the supernatant is indicated by hatching.

The acetone – water extraction.

In the clear region of this graph we find an optimal extraction capacity for acetone containing about 20% water, This is very remarkable, since acetone is considered to be a very poor solvent

for lecithin; possibly the presence of simple lipids promotes the solubility of the phospholipids. This observation raises doubts concerning the frequently-used drying technique with acetone: our experiments indicate that a high loss of complex lipids is not imaginary.

The most interesting finding, however, is the suppression of the extraction capacity in the presence of 50% water. This phenomenon could be exploited when washing of the pre-coagulated substrate is wanted in order to remove water-soluble contaminants or, conversely, for the investigation of the water-soluble substances of yolk powder. Testing this extraction mixture for the extraction of amino acids from yolk powder, it was found that they were extracted while the lipids remain in the yolk powder on the filter.

The alcoholic – water extraction.

The graphs for methanol, ethanol, and tertiary butanol generally show a decrease in extraction capacity with increase in the percentage of water. This phenomenon is less pronounced with tertiary butanol. In our sequence the appearance of the turbidity of the supernatant is shifted to higher percentages of water, indicating that the proteins become less dispersible. This observation is very important for the subsequent investigation described on page 152, dealing with the extraction of fresh material. From these experiments the general conclusion can be drawn that these solvents can be applied with 20% water without causing emulsification.

2. The extraction capacity of water-immiscible solvents.

Examination of Table XXXIII makes it obvious that a great many extraction mixtures could be composed with a solvent of Group A as basis. To limit the combinations, we selected first from this group the solvent combining the highest extraction capacity with the lowest boiling point.

Solvents.

Group A, Table XXXIII.

Method.

One-gram samples yolk powder were weighed into each of 7 small Erlenmeyer flasks and one of the solvents was added by pipette to each sample (2.5 ml). After being supplied with a small glass iron-core stirrer, the flasks were placed on a magnetic stirring device for 30 minutes. The extract was then filtered off rapidly over a sintered glass filter. After all extracts were collected, their phospholipid content was analyzed by paper chromatography. (Appendix page 138).

Results.

The chromatogram in Fig. 72 shows the results of the extractions in the same sequence as listed in Table XXXIII.

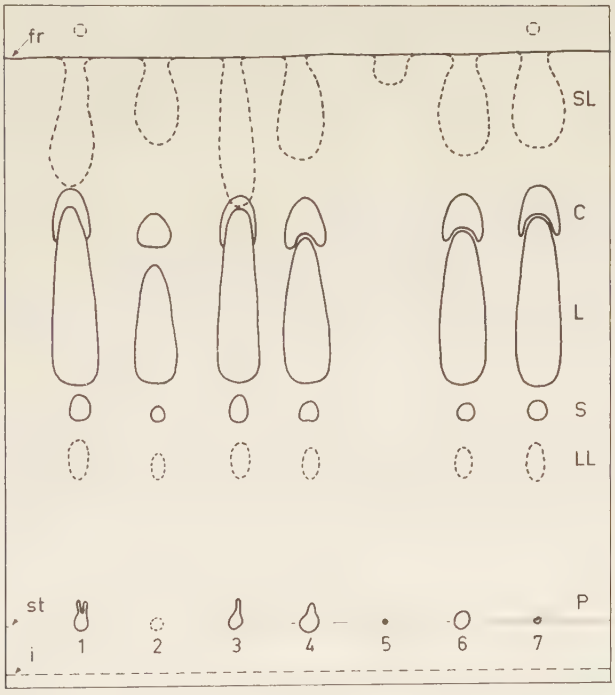

Fig. 72. Chromatogram showing the composition of extracts of yolk powder with various organic solvents. 1-diethyl ether; 2-petroleum ether; 3-chloroform; 4-carbon tetrachloride; 5-ethyl acetate; 6-benzene; 7-iso-butanol. SL-simple lipids; C-cephalin; L-lecithin; S-sphingomyelin; LL-lysolecithin; P-protein.

The effectiveness of the extraction was estimated by measurement of the lecithin spot area; the values are given in Table XXXIV.

Table XXXIV.

solvent	lecithin spot area in cm^2
diethyl ether	9.8
petroleum ether	5.9
chloroform	8.9
carbon tetrachloride	8.0
ethyl acetate	—
benzene	9.1
iso-butanol	9.8

Remarkably, ethyl acetate proved to be a very poor extraction liquid for both phospholipids and simple lipids; only a faint spot could be detected at the solvent front of the chromatogram. Arranging the solvents according to their extraction capacity, we get the following series: diethyl ether, iso-butanol, benzene, chloroform, carbon tetrachloride, and petroleum ether; chloroform and benzene gave slight emulsification.

Conclusion.

It is evident from this series that diethyl ether was the choice for further work, since this solvent combines the greatest extraction capacity with the lowest boiling point.

3. The influence of water-miscible solvents on the extraction with diethyl ether.

Considering only the water content of the yolk powder (50%), we can conclude from the results of the first section that is must be possible to perform the preparatory extraction on a scale with a ratio of egg yolk to solvent of 1 : 2½ without complications caused by the proteins. We next studied the influence of acetone, methanol,

ethanol, and tertiary butanol, on the diethyl ether extraction as compared to that of the pure solvents and their mixtures in a volume ratio of 1 to 1. This can be pictured schematically as a triangle with the alcohols placed at the top, as shown in Fig. 73.

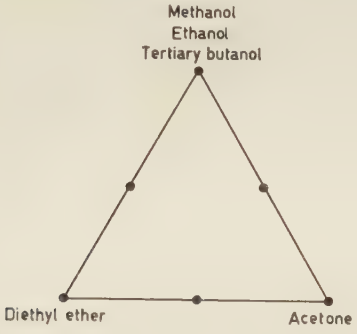

Fig. 73. Triangle showing the pure solvents and mixtures used for the extraction of yolk powder.

Method.

A five-gram sample of yolk powder was weighed into a 50 ml Erlenmeyer flask provided with a ground-glass stopper, and 12.5 ml of the extraction liquid to be investigated was added. The flask was closed after insertion of an iron-core glass stirring rod, and placed on a magnetic device for 30 minutes, after which the liquid was separated over filter paper on a Büchner funnel. The yolk powder remaining on the filter paper was extracted once more in the same way with an extraction liquid of the same composition ,and again separated by filtration. A third extraction of the remaining powder was then performed with 12.5 ml methanol - chloroform, 1 : 1 by volume. This method provides 3 extracts which were chromatographed without dilution, followed by staining as described on page 140. The results are shown in Fig. 74.

Results.

The four chromatograms in Fig. 74 represent the results of the extractions performed with the liquids as indicated in Fig. 73. Each extraction procedure includes 3 phases, consisting of two extractions with a given liquid followed by a third general control extraction with methanol – chloroform. In Table XXXV we give the values of the measurements of the lecithin spot areas.

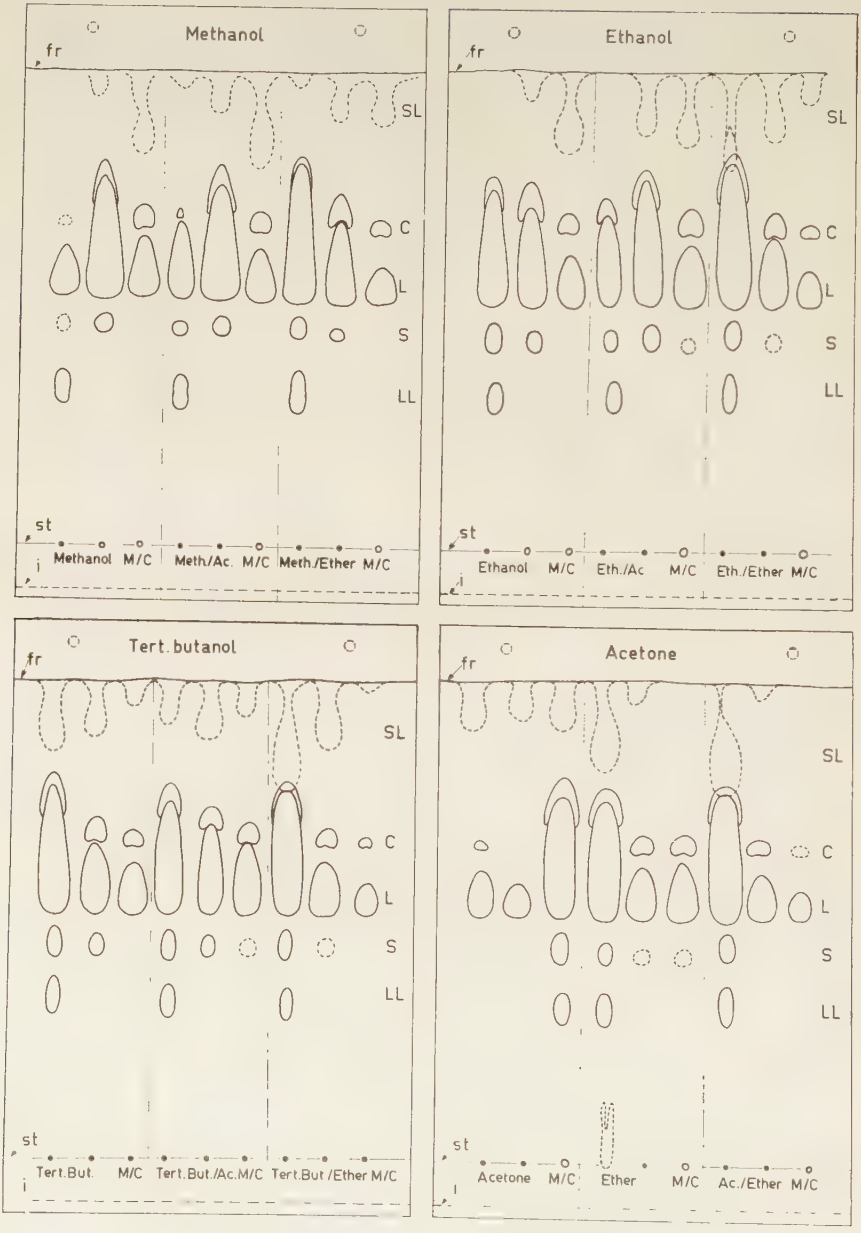

Fig. 74. Chromatograms showing the results of the extraction of yolk powder with different solvent combinations. The vertical broken lines divide the chromatograms into parts representing the extraction of one sample each. The extraction to the right was done with methanol – chloroform, 1 : 1, v/v (M/C). SL-simple lipids; C-cephalin; L-lecithin; S-sphingomyelin; LL-lysolecithin; P-protein. Solvent: diisobutylketon – propionic acid – acetic acid – water, 40 : 10 : 10 : 3, by vol.

Table XXXV.

Extraction liquid	Lecithin spot area in cm²		
	Extr. I	Extr. II	Extr. III (M/C)
Methanol	2.1	6.8	2.5
Methanol-Acetone	3.0	6.5	2.6
Methanol-Diethyl ether	6.4	3.7	2.2
Ethanol	5.2	4.7	2.2
Ethanol-Acetone	3.4	5.7	3.1
Ethanol-Diethyl ether	7.8	3.1	1.4
Tertiary butanol	6.3	3.3	2.5
Tert. Butanol-Acetone	5.2	3.6	3.2
Tert. Butanol-Diethyl ether	6.8 *)	2.7	1.2
Acetone	2.1	1.5	6.7
Diethyl ether	6.6	2.5	2.9
Acetone-Diethyl ether	7.4 *)	2.2	1.0

*) Area not fully expanded.

In interpreting the area of the spots in concentration, we must be aware that some lecithin spots of some first extractions interfere with the large spots of the simple lipids at the front, preventing full expansion of the lecithin spot and suggesting a smaller concentration of this component. The effectiveness of the first two identical extractions in each set of three can be determined by comparing the spot areas of the last identical extractions. Arranging the extraction mixtures according their effectiveness we find:

diethyl ether – acetone,
diethyl ether – tertiary butanol,
diethyl ether – ethanol,
diethyl ether – methanol.

The first extractions of the pure solvents can be arranged in the following sequence:

diethyl ether,
tertiary butanol,
ethanol,
methanol,
acetone.

Comparing these two series we observe, besides the great similarity a remarkable difference; the combination of the most effective pure solvent with the least effective one gives an extraction liquid with the highest yield.

Before introducing the diethyl ether – acetone mixture we studied the extraction capacity of this mixture in relation to its mixing ratio using the same technique as described above. In Table XXXVI we give the values of the measured lecithin spots.

Table XXXVI.

Acetone	Diethyl ether	Lecithin spot area in cm^2
100.00	—	2.0
83.33	16.67	2.7
66.67	33.33	7.7
50.00	50.00	8.5
33.33	66.67	7.5
16.67	83.33	6.9
—	100.00	6.4

Conclusion.

Pre-coagulated material can be extracted in a very efficient way by a diethyl ether – acetone mixture in a material – solvent ratio of 1 : 2.5 (w/v).

It appeared that the same method could be aplied to pre-coagulated beef spinal cord, liver, and kidney.

B. DEVELOPMENT OF AN EXTRACTION PROCEDURE FOR FRESH MATERIAL.

In the extraction of lipids from fresh material the disturbing effects of the proteins are encountered. These difficulties are enhanced when an extraction procedure with relatively small amounts of liquid is used. In this study we investigated whether the property of alcohols to coagulate proteins in solutions could be used in the

extraction procedure for fresh material. On the basis of the results of the preceding study, we took diethyl ether as basis extraction liquid.

Homogenization of the fresh material.

After trying various techniques, it was found that the substrates could best be homogenized in diethyl ether. To avoid evaporation, the extraction was performed in the refrigerator room. The fresh egg yolk or tissue was mixed in a Waring blender with diethyl ether in the ratio of 4 : 5, w/v. This method gave good homogenization of the substrate and the resulting ether - homogenate was used in the following investigation.

1. The effect of water-miscible solvents on the ether – homogenate of fresh egg yolk.

Solvents.

Acetone, methanol, ethanol, isopropanol, propanol, and tertiary butanol.

Material and method.

The shell of an egg is broken and the total content put into a beaker flushed with tap water, in this way the yolk is preserved intact and the white is readily washed away. The yolk (ca. 20 gr.) is then slid into a small funnel and, after drying with filter paper, poured into the mixing glass filled with 25 ml of diethyl ether. After mixing for 5 min. (at 4° C), 25 ml of one of the listed solvents is added and mixing continued for 15 minutes. The mixture is then filtered over filter paper on a Büchner funnel.

For this comparitive investigation, an ethereal stock solution was made with ten eggs, a quantity corresponding to the yolk of one egg in 25 ml diethyl ether being poured into the mixing glass.

For the blank we added 25 ml diethyl ether to the ether-homogenate.

Observations.

The extract of the blank could not be filtered because the filter was obstructed by a jelly-like mass produced by the action of diethyl ether on the proteins.

Except for the filtrate of the ether - tertiary butanol extraction, all the filtrates were more or less turbid due to finely-coagulated protein that passed through the filter. Only with tertiary butanol is the

coagulum coarse enough to be filtered without disturbances. On standing, the turbidity settled and the clear supernatant could be chromatographed.

Results.

After chromatographing and staining (Appendix page 138) the lecithin spots were outlined and their areas measured. The values of these measurements are given in a block diagram in Fig. 75.

Comparison of these results with those of the extraction of

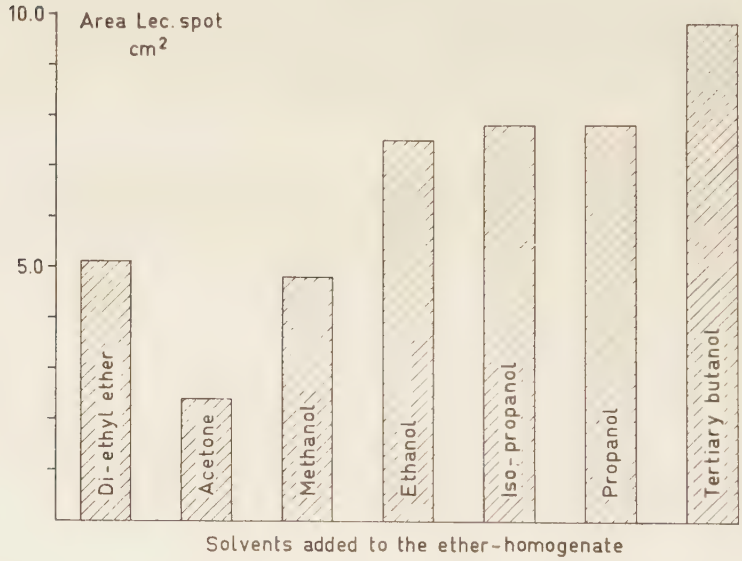

Fig. 75. Diagram of the areas of the lecithin spots, measured after paper chromatography of the extracts obtained with different solvents acting on a ether-homogenate of fresh egg yolk.

powdered egg yolk given in Table XXXV reveals a very striking finding; the ether - acetone extraction gives very poor results with fresh material. This observation emphasizes the importance of the pretreatment of the substrate for the extraction. Previous heating and freezing cause a striking alteration in the extraction capacity of diethyl ether – actone. The excellent results with the combination

diethyl ether – tertiary butanol were expected from the foregoing experiments (see Table XXXV), but it is very remarkable that this combination is twice as effective as diethyl ether – methanol.

2. The extraction of spinal cord, liver, kidney, and blood samples with diethyl ether – tertiary butanol, 1 : 1.

Material.

Yolk of hen's eggs; beef spinal cord; beef liver and kidney; and blood samples from a cow, a horse, and a sheep.

Method.

Samples of 40 grams (in the case of blood, 40 ml) are homogenized in a Waring blender for 5 minutes with 40 ml diethyl ether in the refrigerator room, after which 60 ml of a mixture containing 50 ml tertiary butanol and 10 ml diethyl ether is added and mixing continued for another 15 minutes. (The diethyl ether is added to the tertiary butanol in order to prevent crystallization of this solvent.) The extract is then filtered, using filter paper on a Büchner funnel. In order to determine the efficiency of the extraction we repeated this procedure two times with the residue on the filter paper. This gave three extracts from each substrate which could be chromatographed for their phospholipid content.

Results.

The extraction technique was found to be applicable to all these substrates, irrespective their different composition and origin. The more solid tissues were completely homogenized with diethyl ether and the coagulation of the proteins was in all cases coarse enough to permit rapid and clear filtration, even with the whole blood samples containing a very high percentage of water.

Since a wide variety in the concentration of the different extracts could be expected, they were spotted on a test strip and, after staining, visually interpreted. The results led us to dilute the yolk extracts two times, while the concentration of the blood extracts had to be raised eight times in order to get a chromatogram with distinct spots. Fig. 76 shows the chromatogram of the first extractions.

Apart from differences in quantity and the occurrence of other components in small amounts, the qualitative composition of these different materials shows great similarity. The main components in

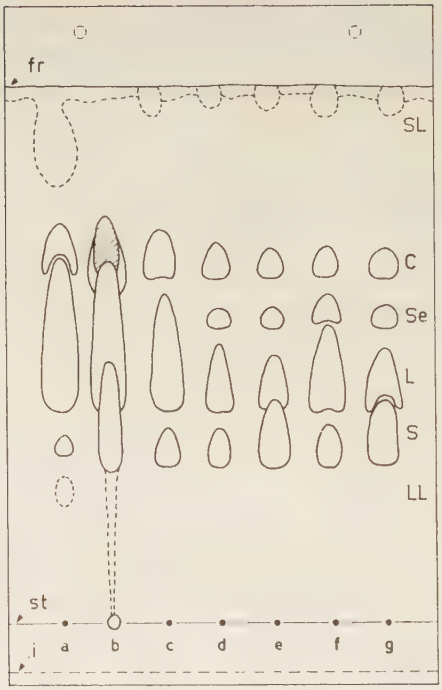

Fig. 76. Chromatogram of diethyl ether – tertiary butanol extracts of: yolk of hen's egg (a); beef spinal cord (b); beef liver (c); beef kidney (d); blood samples of a cow (e), a horse (f), and a sheep (g). SL-simple lipids; C- cephalin; Se-phosphatidyl-serine; L-lecithin; S-sphingomyelin; LL-lysolecithin.

all extracts are lecithin, cephalin, and sphingomyelin; some samples also contain small amounts of phosphatidyl-serine and lysolecithin.

The chromatograms of the second and the third extractions are not reproduced because only small, faint spots of the main components could be detected, indicating that, roughly estimated, about 80 to 90% of the total amount of the lipids is extracted initially.

It appeared that this concentrated extraction technique, perhaps with slight modifications, could be employed for quantitative estimations of the total lipid content of fresh biological materials.

II. THE ISOLATION METHOD.

The isolation of the phospholipids from the other extracted substances was performed by column chromatography. The experi-

mental work was carried out mainly with the concentrated total extract of yolk powder. This extract contains a large amount of simple lipids which interfere with the adsorption of the phospholipids on the column. Using this unfavourable substrate for the development of a suitable isolation method, it must be possible to apply the same method to less contaminated phospholipid mixtures.

a. Selection of the adsorbent.

In the literature on column chromatography in the field of lipid research, the preferential use of two adsorbents is found: the oxides SiO_2 and Al_2O_3. We chose Al_2O_3 (Merck's aluminium oxydatum standard.), because this adsorbent shows the following advantages over SiO_2:

1. The phospholipids are more strongly bound to the column, enabling better separation from the contaminants.
2. Higher adsorption capacity for the phospholipids; this property reduces the column to manageable dimensions.
3. Higher flow rate.
4. Acid components, such as fatty acids, are very strongly fixed on the column.

b. **Apparatus.**

We used two apparatusses, a small column (up to 25 gr adsorbent) and a larger one (up to 250 gr adsorbent) for the preparation of larger amounts, both with the same shape. This chromatographic equipment is shown in Fig. 77.

Apparatus I consists of three parts: the cylindrical column B with a spherical widening A at the top as reservoir for the eluens, and at the bottom the outlet capillary C. To avoid drainage of the column, special care was used in designing the outflow. The outlet capillary is elongated and bent upwards in an S-shape, reaching above the level of the filled column. The column volumes are 25 and 250 ml respectively. The reservoirs at the top of the columns have volumes of about 60 ml and 600 ml. Apparatus II is a pressure bottle provided with a mercury manometer in order to apply constant pressure. Apparatus III is used as falling tube for the preparation of the column, when mounted on I. Apparatus IV is used for the application of the extracts to the column.

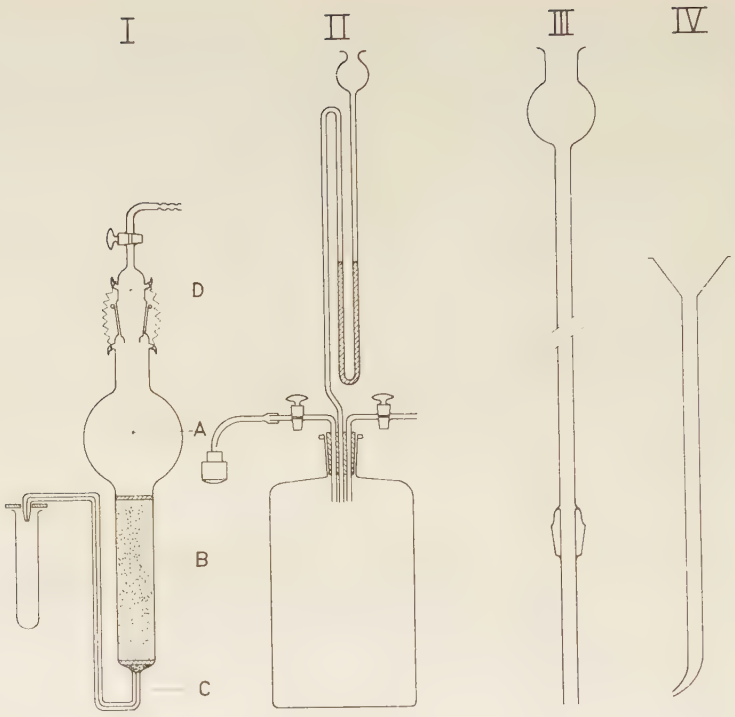

Fig. 77. Apparatus used for column chromatography. For description, see text.

c. **Filling of the column.**

Good packing of the column is essential for good chromatographic separation. Irregularities such as channeling or air bubbles will disturb the formation of separated zones. These disturbances were avoided in the following way. Column I is mounted exactly vertically on a support, the outlet is closed and the column filled half full with the solvent. A piece of glasswool is then placed on the outlet capillary, and on this purified sea sand is poured in a horizontal layer about 5 mm thick. Falling tube III is then mounted on column I, and both column and tube are filled with the solvent. Into the falling tube is poured the weighed Al_2O_3 which whirls downwards through the solvent, building up a very regular column on the sea sand bed. After settling, the column is covered wih a thin layer of sea sand to stabilize the upper surface. Then the falling tube is replaced by a perforated ground-glass stopper D, provided with a tap and connected by a tube to pressure bottle II. Next, the capillary is opened and the excess of solvent is allowed to flow off. The column is then ready for use.

d. Guiding principles for the selection of the elution liquids.

The choice of the solvents used in chromatography is often a matter of trial and error. However, in the case of the column chromatography of lipids a very useful study has been published by Trappe (1940), who investigated the "adsorption value" of different lipids from different solvents with different adsorbents. (Adsorption value is the quantity of a lipid, in percentages, taken up from a solvent by an adsorbent under standard conditions.) On the basis of these data he arranged the solvents in a series with decreasing elution strength, i.e. independent of adsorbent and lipid, each solvent of this "eluotropic" series is a better eluens for an adsorbed lipid than the following solvent (see Table XXXVII).

Table XXXVII.

Adsorption values related to the solvent and the adsorbent (according to Trappe, 1940).

Solvent	Aluminum silicate			Silicic acid			Al_2O_3 acc. to Brockmann		Kaolin
	oleic acid	cholesterol	stearic chol. ester	oleic acid	glyceryl trioleate	palmitic chol. ester	chol.	acetic chol. ester	chol.
methanol	0.0	~	~	0.0	~	~	0.0	~	~
ethanol	32.3	~	~	0.0	0.0	~	4.4	~	~
n-propanol	44.6	~	~	0.0	0.0	~	2.8	~	~
acetone	46.8	~	~	0.0	0.0	~	3.8	~	~
ethyl acetate	71.9	0.0	0.0	0.0	0.0	0.0	14.8	0.0	1.3
diethyl ether	80.9	0.0	0.0	0.0	0.0	0.0	63.1	0.0	0.0
chloroform	88.9	0.0	0.0	69.7	42.8	0.0	24.5	0.0	0.0
methylene chloride	89.8	0.0	0.0	71.8	54.4	0.0	53.2	0.0	0.0
benzene	98.2	75.7	0.0	88.2	76.5	0.0	95.1	11.2	40.0
toluene	98.0	78.6	0.0	90.6	86.9	0.0	97.0	12.8	46.8
trichloro-ethylene	98.6	86.3	0.0	97.4	98.5	33.4	96.3	22.4	—
carbontetrachloride	98.8	96.4	21.6	98.6	100.0	74.6	98.0	53.1	82.1
cyclohexane	100.0	99.2	85.9	100.0	100.0	93.2	100.0	96.8	94.9
petroleum ether b.p. 30-50	100.0	100.0	94.0	100.0	100.0	99.0	100.0	97.9	98.1

Trappe drew attention to the fact that the elution capacity of a solvent of the eluotropic series is proportionally related to its affinity to the adsorbent indicated by the heat of adsorption.

Table XXXVII indicates that there are different ways to elute the components of a lipid mixture from the column.

Firstly, this may be done by a flow of a pure solvent in which the various substances move at different rates according their different binding forces to the adsorbent.

Secondly, we can compose an elution scheme with a mixture of two solvents of different elution strength e.g. methanol – petroleum ether, by increasing this ratio, the elution strength of the mixtures increases and a type of gradient elution is obtained. This sort of elution has one great disadvantage: the so-called "tailing" effect may appear and the zones overlap each other.

Thirdly, we can make use of sudden increments in elution strength, e.g. petroleum ether, chloroform, methanol. This last type of elution (frontal elution) has many advantages:

1. The column is cleared rapidly of a certain substance or mixture of substances, set free from the adsorbent by the sudden increment of the elution strength of the solvent which prevents subsequent re-adsorption on the column.
2. The "tailing" effect does not occur.
3. The amounts of elution liquids required are small.

In addition to the quicker preparation, the eventual deterioration of the adsorbed substances will be limited since the time of adsorption is shorter. In deriving a frontal elution scheme from Trappe's eluotropic series, it must be kept in mind that this sequence holds only when the lipid removed from the adsorbent by a solvent, is soluble in the mobile phase. For column chromatography of phospholipids it is clear that this solubility factor cannot be ignored, e.g. acetone shows a great affinity for the adsorbent but is a poor eluens for the phospholipids because it is a poor solvent for these lipids. This example is chosen intentionally; since it shows us a way to handle the total lipid extract, it must be possible to elute the contaminating simple lipids from the column with acetone, leaving the phospholipids behind. Taking into account the role of the solubility factor in the eluotropic series, it seemed possible to separate substances by creating a scheme for selective frontal elution.

1. **The choice of the water content of aluminum oxide.**

Brockmann and Schodder (1941) and Müller (1945) reported that the adsorption capacity of Al_2O_3 depends greatly on its water content (4% water reduced the adsorption capacity to 5%). In view of this, we started with the study of the adsorption capacity for phospholipids in relation to the water content of Al_2O_3. From Table XXXVII we selected petroleum ether as column-preparing solvent and as eluens, since Al_2O_3 shows the highest adsorption value in combination with this solvent.

Apparatus.

Small column.

Adsorbents.

About 200 gram of Merck's aluminum oxide was heated for 120 hours at 120° C. After cooling in a dessicator, 5 ml water was added to 95 gram of this prepartion, and the mixture was shaken for 70 hours. By taking different ratios of the dried and the moistened Al_2O_3, six samples of 20 grams were prepared with a water content ranging from 0 to 5 percent.

Solvent.

Petroleum ether, b.p. 60°-80° C.

Distribution liquid.

The residue of the yolk-powder extract obtained after evaporation of the extraction liquid gave, with petroleum ether, a slightly turbid solution which could be cleared by adding an equal volume of acetone.

Chromatography.

Just above the upper level of the column, 10 ml of a solution of yolk-powder extract (corresponding to the extract of one egg yolk) in petroleum ether and acetone (1 : 1, v/v) was introduced into the

column. The glass wall and upper level of the column were rinsed twice with 1 ml of the same mixture of petroleum ether – acetone. The elution was then started with 60 ml petroleum ether. The collected fractions of each column were checked for their phospholipid content by paper chromatography.

Results.

It appeared that the adsorption capacity of Al_2O_3 for phospholipids is highly dependent on its water content. The best results were obtained with a column containing 3% water, although the collected fractions still contained phospholipids.

The unfavourable results with totally dry Al_2O_3 are explained by the supposition that the phospholipids, in the absence of any water, tend to associate with their hydrophilic groups together (probably di-complexes) and their hydrophobic parts sticking outwards. In this association the phospholipids behave as entirely hydrophobic substances and are therefore readily eluted together with the simple lipids. The poor results with a water content of 5% are understandable in view of the eluotropic series of Trappe.

The adsorption capacity for triglycerides appeared to be independent of the water content of the Al_2O_3; they were always readily eluted in the first fractions. The adsorption capacity for the coloured substances was highest with the dried Al_2O_3, but was subsequently reduced when the water content was increased.

2. The adsorption capacity of Al_2O_3 (3% water) with acetone as column-preparing solvent and as eluting solvent.

On the basis of the considerations on page 160, in this investigation we used the combination of the most favourable Al_2O_3 preparation (water percentage 3%) with acetone as preparing solvent and as eluens. The small column was used with 20 grams of Al_2O_3.

Chromatography.

Ten ml of a solution of yolk-powder extract dissolved in petroleum ether - acetone, (1 : 1, v/v) was applied to the column as described on page 161. Chromatography was then started with 60 ml acetone; it

appeared that no phospholipids were eluted. Since some coloured substances remained on the column, chromatography was continued with 60 ml acetone containing 2% water. With this eluens the column was cleared of pigmented substances, and the phospholipids remained totally on the column.

Conclusion.

The adsorption system of humidified aluminum oxide - acetone is excellent for the separation of the phospholipids from an egg yolk extract dissolved in petroleum ether – actone. The contaminating substances can be readily removed by elution with acetone containing 2% water.

3. **General application of the separation technique.**

This technique could also be employed on a large scale for the separation of greater amounts of phospholipids from extracts derived from many sources; fresh egg yolk, commercial egg lecithin, commercial soy lecithin, rat liver, beef spinal cord, etc.

The large column was used with 250 grams of Al_2O_3 (3% water) and acetone as preparing solvent. After introduction of the dissolved extract into the column, the contaminants were eluted with 700 ml acetone containing 2% water. However, some precautions must be taken for the distribution solvent. The concentrated extracts must be applied to the column as a clear solution. It was found that some extracts gave no clear solution with petroleum ether – acetone, 1 : 1, v/v. In the case of the extracts from commercial soy bean lecithin and rat liver, the turbidity could be removed by previous washing of the extract with water (see below), in the case of the extract from beef spinal cord, another distribution solvent had to be taken (chloroform).

a. Removing of water-soluble substances from the extract.

After extraction of the material with the appropriate extraction liquid (diethyl ether – acetone or diethyl ether – tertiary butanol) the extract was separated from the solids by filtering over a Büchner

funnel mounted via a connecting tube carrying a small side arm for connection to a filter pump.

Prior to filtration, an amount of water is added to the separatory funnel corresponding to half the volume of the extraction liquid used. After filtering of the extract, the funnel can be shaken directly when diethyl ether – tertiary butanol is used as extraction liquid. When diethyl ether – acetone is used as extraction liquid, petroleum ether is added previously to an amount of half a volume of the extraction liquid, in order to obtain a good separation into two layers after shaking. After the formation of two layers, the supernatant was collected and evaporated in nitrogen atmosphere in a rotating vacuum evaporator.

b. Chloroform as distribution liquid.

It was found that the extract of beef spinal cord could only be dissolved in chloroform. To avoid difficulties, the following points must be considered. Owing to its higher density, chloroform tends to replace the acetone in the upper part of the column. This acetone dissolves in the cloroform of the extract still situated above the sea-sand level of the column, and may cause a precipitation which blocks the further development of the chromatography. Therefore, the following technique must be applied. Prior to the application of the extract to the column, the upper acetone layer in the column is replaced by chloroform (small column 2 ml, large column 5 ml). The spinal cord extract is introduced into the column very rapidly under high pressure. Then the upper level is washed twice with a small amount of chloroform, followed by chromatography with acetone containing 2% water.

III. THE PURIFICATION METHOD.

For the purification of the phospholipids we started with the part of the total lipid extract remaining on the column after elution of the contaminants with 2% aqueous acetone. In 1957 Lea and Rhodes described the fractionation of phospholipids on aluminum into two groups: choline-containing phospholipids (e.g. lecithin) eluted from the column with methanol-chloroform (1 : 1, v/v) and noncholine-containing phospholipids (e.g. cephalin) eluted with ethanol-chloro-

form-water (5 : 2 : 2, by volume). In view of the work of Trappe, this elution scheme must be understood as follows: in both mixtures, chloroform serves principally as solvent for the phospholipids removed from the adsorbent by the action of methanol or ethanol-water. The higher elution strength of the latter is caused by its water content. Since aluminum acts as a positive adsorbent, it is clear that cephalin is bound more strongly than lecithin owing to its lower iso-electric point.

The description of the preparation of the various phospholipids is brief; a detailed description of the experiments leading to the final technique is omitted.

1. Lecithin preparations.

It appeared that in our technique we could employ methanol-chloroform (1 : 1, v/v) after prior elution of the contaminants with 2% aqueous acetone. The choline-containing phospholipids were eluted rapidly without contamination of choline-free phospholipids. Depending on the phospholipid composition of the extracted material, the fractions contain either lecithin in a pure state or lecithin more or less contaminated with other choline-containing phospholipids (sphingomyelin, lysolecithin). Practically the whole amount of lecithin could be eluted from the column by taking 2.5 ml of eluens for each gram of aluminum oxide.

a. Egg yolk lecithin.

The first methanol-chloroform fractions of an extract of egg yolk contain lecithin in a practically pure state. The following fractions contain, in addition to large amounts of lecithin, small amounts of sphingomyelin and lysolecithin. For the bulk preparation of lecithin from hen's eggs we accepted this small amount of other choline-containing substances (about 5%). The area of the spot on the chromatogram gave the impression that the percentage of lysolecithin is lower when the extract is made from fresh egg yolks instead of yolk powder.

b. Soy bean lecithin and liver lecithin.

The lecithin preparation from commercial soy bean lecithin and from fresh rat liver were obtained in a very pure state with methanol-chloroform; only traces of choline-containing substances could be detected.

c. Spinal cord lecithin

The preparation of lecithin from spinal cord is complicated by the occurrence of large amounts of sphingomyelin. To obtain lecithin in a pure state we developed another elution scheme based on the insolubility of sphingomyelin in diethyl ether. Using this solvent as mobile phase in combination with solvents acting as eluens, it appeared to be possible to elute lecithin while sphingomyelin remains on the column.

After elution of the pigmented contaminants with 2% aqueous acetone, lecithin could be eluted without sphingomyelin with diethyl ether – acetone – tertiary butanol – water (15 : 10 : 5 : 2, by volume). However, a paper chromatographic check showed that the first lecithin fractions contained another substance which was situated above the lecithin spot. This substance was not stained but was detected as a white spot on putting the chromatogram in the Acid Fuchsin staining bath. After storage at 4° C these first fractions became gelatinous. It was found that this contamination could be removed by diethyl ether – acetone – tertiary butanol – water (15 : 10 : 5 : 1, by volume); the lecithin remained on the column. Lecithin is then eluted by increasing the water content of the eluens: diethyl ether – acetone – tertiary butanol – water (15 : 10 : 5 : 2, by volume). The collected fractions were chromatographed and stained, followed by the measurement of the areas of the outlined spots with a planimeter.

The values of these measurements in square cm are taken as ordinates in Fig. 78; along the abscissa the corresponding tube number of the fraction is indicated. The last lecithin fractions contain a small amount of sphingomyelin caused by a critical saturation of the column with water. For a pure lecithin preparation it is preferable to take tubes 47 to 70.

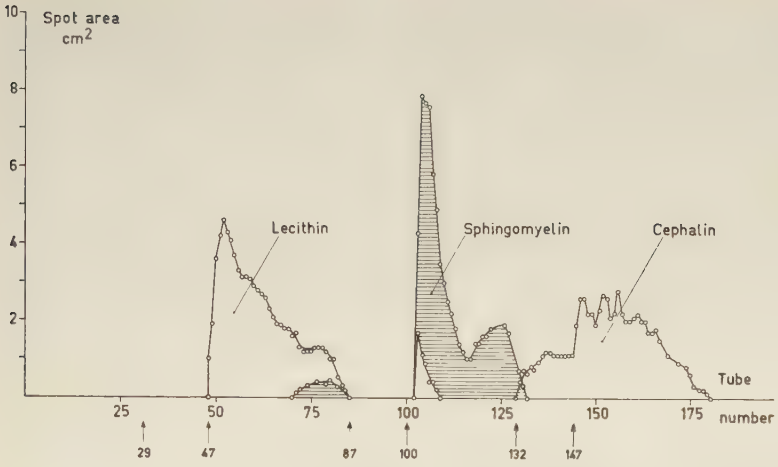

Fig. 78. Diagram of the fractionation of a diethyl ether - tert. butanol extract from beef spinal cord over an Al_2O_3 column. The collected fractions were analyzed by paper chromatography and the areas of the phospholipid spots were measured. The values of these measurements are plotted against the corresponding fractions.

Solvents:

Tubes 0- 29 acetone (containing 2% water)
29- 47 diethyl ether – acetone – tert. butanol – H_2O (15 : 10 : 5 : 1)
47- 87 diethyl ether – acetone – tert. butanol – H_2O (15 : 10 : 5 : 1)
87-100 acetone
100-132 methanol – chloroform (1 : 1)
132-147 ethanol – chloroform – H_2O (5 : 2 : 1)
147-182 ethanol – chloroform – H_2O (5 : 2 : 2.8)

2. Sphingomyelin preparation.

For the preparation of sphingomyelin, spinal cord is preferred although other sources can also be used, for example sheep erythrocytes. The preparation technique was developed in connection with the elution of lecithin from the aluminum column as described for the preparation of spinal cord lecithin.

The elution of lecithin is performed with a mixture containing about 6% water, which reduces the capacity of the adsorbent and causes and increased effect of the eluens applied subsequently. For example, applying methanol - chloroform (1 : 1, v/v) to this column

for the elution of sphingomyelin, the more strongly-bound cephalin fraction is eluted too. Before elution of sphingomyelin in a pure state from the column, the adsorbent must be restabilized by diminishing the water content. This was done by washing the column with dry acetone. After this intermediate drying procedure, sphingomyelin could be eluted with methanol – chloroform (1 : 1, v/v) without contamination with cephalin. The collected fractions were chromatographed, followed by staining and measuring of the areas of the spots; these values are plotted in Fig. 78. In the first tubes we observed a contamination with a small residual amount of lecithin, i.e. about 5% of the total amount of sphingomyelin.

3. **Cephalin preparations.**

a. Egg yolk cephalin.

The preparation of this cephalin was performed after the previous elution of lecithin from the column. Applying the eluens mixtures used by Lea, Rhodes, and Stoll (1955) (ethanol – schloroform – water, 5 : 2 : 2, by volume), the cephalin fraction contained coloured substances and the rest of the choline-containing group. To avoid this contamination we cleared the column from these substances with ethanol – chloroform – water (5 : 2 : 1, by volume).After this prior clearing, the cephalin is eluted in a pure state from the column with a stronger eluens consisting of ethanol – chloroform – water (5 : 2 : 2.8, by volume).

b. Spinal cord cephalin.

The cephalin preparation from spinal cord is performed after the elution of sphingomyelin from the column. It was found (see Fig. 78) that ethanol – chloroform – water (5 : 2 : 1, by volume) already acts as an eluens for cephalin, undoubtedly caused by the water previously supplied with the solvents used for the elution of lecithin. In the diagram we observe an increase in the eluted amount of cephalin after increasing the percentage of water in the eluens to: ethanol - chloroform - water, 5 : 2 : 2.8, by volume.

C. THE FINAL METHOD FOR PREPARING PHOSPHOLIPIDS FROM NATURAL SOURCES.

EXTRACTION.

a. Pre-coagulated material.

The material is boiled for 30 minutes in water (weak material can be enclosed in a plastic bag), cut into small pieces, and then frozen for 3 hours at —13° C. At the same low temperature the material is powdered in a Waring blender. To 1 part (gr) of powder are added 3 parts (vol) of the extraction mixture, diethyl ether – acetone, 1 : 1. Mixing is continued for 15 minutes, after which the whole mixture is filtered over a Büchner funnel mounted on a separatory funnel. If necessary, the extract (i.e. from 1 part of material) can be washed with 1½ parts H_2O together with 1½ parts petroleum ether. After separation, the supernatant is collected in a spherical vessel and evaporated under reduced pressure in nitrogen atmosphere. Dependent on the solubility, the residue is dissolved in petroleum ether – acetone or chloroform.

b. Fresh material.

One part (gr) of fresh material is homogenized for 5 minutes with 1 part (vol) of diethyl ether at 4° C in a Waring blender. To this is added 1½ parts (vol) of a mixture of tertiary butanol – diethyl ether, 5 : 1. Mixing is continued for 15 minutes, after which the whole is filtered over a Büchner filter mounted on a separatory funnel. The diethyl ether – tertiary butanol extract (1 part material) can be washed with 1½ parts (vol) H_2O. After separation, the supernatant is collected in a spherical vessel and evaporated under reduced pressure in nitrogen atmosphere. Dependent on the solubility, the residue is dissolved in petroleum ether – acetone or chloroform.

ISOLATION.

For 1 part of extracted material, 1 part (gr) of Al_2O_3 (moisture 3%) is taken as adsorbent. The column is made up with acetone as

column-preparing solvent. After introduction of the extract into the column, the contaminating substances are eluted with acetone containing 2% water.

PURIFICATION.

Dependent on the lipid composition of the extracted material, the elution of the individual phospholipids is performed as follows:

When the extracted material contains chiefly lecithin and cephalin (hen's egg, soy bean, rat liver), lecithin can be eluted with methanol-chloroform, 1 : 1, v/v. After an intermediate washing of the column with ethanol-chloroform-water, 5 : 2 : 1, (v/v), cephalin can be eluted with ethanol-chloroform-water, 5 : 2 : 2.8, (v/v).

When the extracted material contains sphingomyelin and perhaps other complex lipids besides lecithin and cephalin, the following procedure is employed. In order to elute other contaminating complex lipids (e.g. cerebrosides in the case of spinal cord) the column is previously washed with diethyl ether - acetone - tertiary butanol – water (15 : 10 : 5 : 1, v/v). The lecithin can then be eluted with diethyl ether – acetone – tertiary butanol – water (15 : 10 : 5 : 2, v/v). After an intermediate washing with acetone, sphingomyelin can be eluted with methanol – chloroform (1 : 1, v/v). Cephalin can then be eluted with ethanol – chloroform – water (5 : 2 : 1 and 5 : 2 : 2.8, by vol).

D. LYSOLECITHIN PREPARATION.

For the preparation of lysolecithin we treated lecithin with lecithinase A which, as established by Tattrie (1959) and by De Haas and Mulder and Van Deenen (1960), removes the fatty acids esterified at the β-position of lecithin.

The lecithin fraction prepared from 100 grams of yolk powder is collected in a spherical vessel and the solvent (methanol-chloroform, 1 : 1, v/v) is evaporated under reduced pressure. The practically colourless lecithin residue is then dissolved in 150 ml diethyl ether and 5 ml of 0.1 per cent rattlesnake venom (Crotalus adamanteus) in a 0.1 m phosphate buffer (pH 7.4) is added. After being provided

with a stirring rod, the vessel is closed and placed on a magnetic stirring device. The solution is stirred at room temperature and the conversion into lysolecithin is followed by paper chromatography. After 0, 19, 45, and 168 hours, 0.5 ml samples of the stirred liquid are pipetted into a test tube, the solvent evaporated, and the residue dissolved in 5 ml methanol-chloroform (1 : 1, v/v). The solutions are then chromatographed.

During conversion the original homogenous, slightly turbid solution acquires a milky appearance after one hour; after 45 hours the milky appearance disappears and when stirring is stopped the solution separates into two layers. Analysis of these two layers for phospholipids reveals that the lower layer (¼ of the volume) contains phospholipids but the supernatant contained none (see Fig. 79.

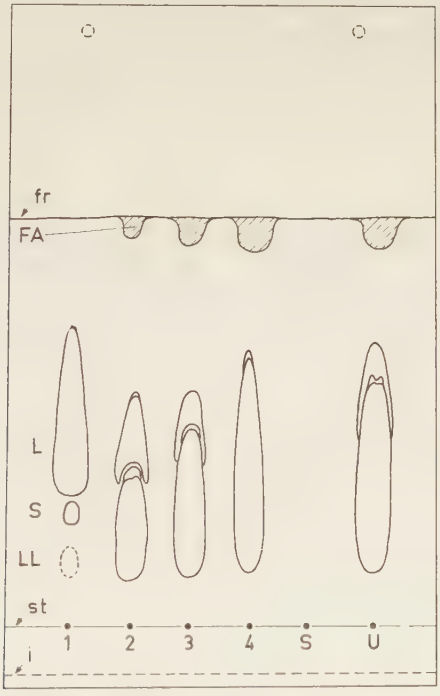

Fig. 79. Chromatogram showing the conversion of lecithin into lysolecithin by the action of snake venom over 168 hours. From left to right: 1, the initial preparation; 2, 3, and 4, after 19, 45, and 168 hours respectively. S is a sample of the supernatant and U a sample of the lower layer of the reaction system which separates out when stirring was stopped after 45 hours of conversion. FA-fatty acids; L-lecithin; S-sphingomyelin; LL-lysolecithin.

which shows a combined chromatogram of the solution after various conversion times).

According to the chromatogram, after 168 hours the conversion is practically complete.

Purification of the lysolecithin.

To obtain a pure lysolecithin preparation, this substance must be separated from the snake venom and the fatty acids split off from the lecithin. For this purpose the total solvent is evaporated under reduced pressure in nitrogen atmosphere. Any residual water is removed by evaporation in combination with methanol. The dry residue is resolved in methanol-chloroform (1 : 1, v/v) and applied to a column prepared with the same solvent and filled with 100 grams of Merck's aluminum oxide. Elution with the same solvent prevents the adsorption of lysolecithin on the column, while the contaminating substances are retained. The first column fractions contain the total pure lysolecithin content.

E. THE PREPARATION OF PHOSPHOLIPID SOLUTIONS OF KNOWN CONCENTRATION FROM COLLECTED COLUMN FRACTIONS.

The column fractions containing a given phospholipid are combined in a volumetric flask which is then brought up to volume with the solvent of the fractions. From this phospholipid solution, samples are pipetted into four small Erlenmeyer flasks of known weight, with wide and absolutely flat bottoms. The flasks are heated in a sand bath (100° C) and after evaporation of the solvent the residue is dissolved in a small amount of diethyl ether and re-heated. This is repeated until a very thin equal film is formed on the bottom. The flasks are then closed with a ground-glass stopper and placed in a dessicator to cool before weighing. Heating, cooling, and weighing are repeated according to a rigid time scheme until a constant weight is obtained. By using the same technique for the empty and the phospholipid-containing flasks, the dry weight of the phospholipid

preparation can be estimated very exactly. From the average of the weights, the amount of phospholipid in the rest of the solution can be calculated. The solvent of this residual amount is evaporated under reduced pressure in nitrogen atmosphere and the residue is dissolved in a calculated volume of 91% tertiary butanol in order to obtain a phospholipid solution of a given concentration.

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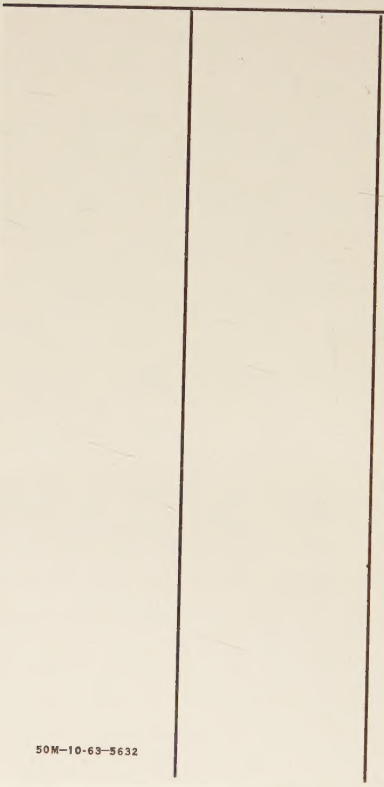

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